12-9-2016

Role of Selenium in Age-Related Degeneration: Selenotranscriptome Hierarchy and Characterization of Selenoprotein H

Lei Cao

Role of selenium in age-related degeneration: selenotranscriptome hierarchy and characterization of selenoprotein H

By

Lei Cao

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Nutrition
in the Department of Food Science, Nutrition, and Health Promotion

Mississippi State, Mississippi

December 2016

Role of selenium in age-related degeneration: selenotranscriptome hierarchy and characterization of selenoprotein H

By

Lei Cao

Approved:

_____

Wen-Hsing Cheng
(Major Professor)

_____

Tung-Lung Wu
(Minor Professor)

_____

Din-Pow Ma
(Committee Member)

_____

Teajo Kim
(Committee Member)

_____

Terezie T. Mosby
(Committee Member)

_____

William B. Evans
(Graduate Coordinator)

_____

George M. Hopper
Dean
College of Agriculture and Life Sciences

Name: Lei Cao

Date of Degree: December 9, 2016

Institution: Mississippi State University

Major Field: Nutrition

Major Professor: Dr. Wen-Hsing Cheng

Title of Study: Role of selenium in age-related degeneration: selenotranscriptome hierarchy and characterization of selenoprotein H

Pages in Study 93

Candidate for Degree of Doctor of Philosophy

Selenium (Se) is an essential trace element exerting its biological functions mainly through selenoproteins. Our lab has recently shown a paradoxical role of dietary Se at nutritional levels of intake in the delay of age-related degeneration while reducing longevity in mice carrying humanized telomeres. The first aim of this dissertation was to evaluate the effects of long-term dietary Se deficiency, aging, and sex on selenotranscriptome hierarchy in tissues. Four unique patterns in selenotranscriptomic changes were summarized. First, the responses of selenotranscriptomes to dietary Se deprivation and aging were sexually dimorphic. Second, a few selenoproteins responded to dietary Se deficiency and aging in parallel. Third, there were selenoproteins up-regulated by aging or dietary Se deprivation. Forth, some selenoproteins, especially those in testis, were upregulated by aging in mice on a Se-deficient diet.

Selenoprotein H (SELH) is ranked low in selenoprotein hierarchy and its expression is tissue-specific and abundant during embryogenesis. SELH is a nucleolar DNA-binding protein with thioredoxin (Txn)-like fold and glutathione peroxidase activity. The known biological functions of SELH include redox regulation and

suppression of cellular senescence and tumorigenesis. The second aim of this dissertation was to study the functional interactions of SELH with other selenoproteins and its protective effects against oxidative stress. A FLAG-tagged plasmid expressing wild-type SELH was constructed. Compared to standard cell culture conditions, additional sodium selenite (Na_2SeO_3) increased SELH expression at protein but not mRNA level when FLAG-SELH was overexpressed. In the absence of supplemental Na_2SeO_3, FLAG-SELH overexpression suppressed the expression of other selenoproteins such as glutathione peroxidase 1 (GPX1) and thioredoxin reductase 1 (TrxR1) at protein but not mRNA level. FLAG-SELH overexpression protected the cells against oxidative stress only when additional Na_2SeO_3 was added. Identification of FLAG-SELH-associated proteins confirmed its nucleolar location.

Altogether, a distinctive set of selenoproteins is maintained under dietary Se deficiency in a tissue- and sex-specific manner during the aging process. While SELH is ranked low in selenoprotein hierarchy implying that it is dispensable, this nucleolar selenoprotein competes with other selenoproteins for Se and protects the cells against oxidative stress.

ACKNOWLEDGEMENTS

I owe the most genuine gratitude to my major advisor, Dr. Wen-Hsing Cheng. His professionalism and reasonableness always make me feel lucky to have him as my advisor and mentor. His guidance and support over the past three years taught me how to think and act like a real researcher. His dedication to his work and his students showed me what a good researcher and professor should be like.

I would like to thank my committee members, Dr. Din-Pow Ma, Dr. Teajo Kim, Dr. Tung-Lung Wu, and Dr. Terezie Mosby for their time and contribution of my work. I want to thank them for letting me use their equipment and knowledge when I need. The professional advice I got from them, no matter in their classes or their labs, let me know how to make my study right and better.

I would like to thank my parents, my sister, my husband Sanggil Lee, and the rest of my family for their support and encouragements. There are always ups and downs during the Ph.D. study and in life. I want to thank them for always being there for me and with me whenever I need.

I would like to thank Dr. Tibor Pechan at IGBB for the LC-MS-MS analysis, Dr. Huawei Zeng in USDA Grand Forks Human Nutrition Center for the Se analysis, and Ryan TY Wu for collecting mouse tissues.

I would like to thank my lab mates Li Zhang and Hsin-Yi Lu, and other friends for giving me their ears and hands when I need their help and time. Meaningful

discussions with them keep me asking questions and trying to solve them. I am very grateful that I made quite a few good friends during my Ph.D. study who treat me like families.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Selenium and Selenoproteins

1.1.1 Biochemistry and metabolism of Se

Se is an essential trace element for maintaining the healthy life of all domains (1). The major food source of Se includes bread, cereal, domestic animal meat, and seafood (1). Se enters the food chain through incorporation into plant proteins. The Se content of food is highly dependent on the amount of Se in the soil and the ability of plants to take up and accumulate Se (2). Usually, when feed crops grow on soils contain less than 0.6 mg total Se kg^{-1}, they may become Se deficiency. Consequently, livestock fed with feed crops containing deficient Se may also become Se deficient (3).

The major dietary forms of Se include inorganic selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), and organic selenocysteine (Sec) and selenomethionine (Se-Met) (2). Regardless of the forms of Se in the consumed food, Se is processed to be selenide (Se^{2-}) and then used for selenoprotein synthesis (4). Moderately excessive Se can be detoxified through sequential methylation into dimethylselenide, which is then excreted into the breath, or selenosugars and trimethylselenide, which are excreted into the urine (2).

The absorption of Se occurs mainly in the lower part of the small intestine by different routes and mechanisms, which are usually shared with their sulfur analogs (2). Absorption of almost all forms of Se, inorganic as well as organic, is generally believed

to be efficient (about 70% - 90% absorption). Healthy individuals having a balanced and varied diet should acquire sufficient amount of Se at nutritional level, therefore they do not need to supplement Se to a level above the nutritional needs (4).

Se supplementation is recommended when the intake from food is low. There is a very narrow range between deficiency, essentiality, and toxicity for Se (5). Therfore, those who consider Se supplementation need to be carefully not to reach a toxic level. Regarding human requirement, regular consumption of food containing less than 0.1 μg Se g^{-1} results in deficiency, while consumption of food containing more than 1 μg Se g^{-1} may cause toxicity (5).

Because the need of Se for healthy adults is only 55 μg per day, human Se deficiency with obvious clinical signs is not common nowadays. The first human Se deficiency was found in Keshan in Northeast China where soil Se is very low. The Se deficiency syndrome is thus known as Keshan disease, an endemic cardiomyopathy (6). Asymptomatic low Se status has also been reported in residents of New Zealand, Finland and areas of the Eastern United States (1) whose body Se status are not critically low to display overt dificiency symptoms. Nonetheless, government in these regions has taken Se supplementation schemes, including enriched food products, soil fertilizer and/or direct supplementation regimens (1). In contrast to Se deficiency, the chronic intake of high levels of Se could cause selenosis (chronic toxicity), which is characterized by loss of hair and nails, garlic breath, and skin lesion (6). The soil in Enshi, China contains exceptionally high level of Se, and Enshi even has the only known Se mine in the world. Ironically, selenosis case studies in Enshi offer data that help nutritionists to determine the upper level of safety Se intake (7, 8)

Serum Se concentration is a convenient marker widely used to assess body Se status (9). A U-shaped link was found between serum Se concentration and all-cause mortality rate. Increasing serum Se concentration up to about 135 μg/L is associated with decreased mortality. When serum Se concentration is higher than 135 μg/L, the higher serum Se concentration correlates to higher all-cause mortality rate (10).

Besides the increased rate of mortality, low Se status has also been associated with poor immune function, cognitive decline, decreased male reproductive performance, and higher risk of autoimmune thyroid diseases (10). The studies of Se roles in cancer risk have been inappropriately designed and studied, thus producing confusing results incomprehensible even for some Se experts. Benefits of higher Se status on reducing the risk of cancers, especially prostate, lung, colorectal, and bladder cancers have usually been observed from observational studies (11). Findings from randomized controlled trials support such a benefit based on the Nutritional Prevention of Cancer Trial that uses selenized yeast containing various forms of Se (12). The Selenium and Vitamin E Cancer Trial (SELECT) used Se-Met as the sole source of Se failed to show cancer prevention in the cohort study 5 years entering the trial when this study was prematurely terminated due to increased risk of type 2 diabetes, alopecia, and dermatitis due to Se supplementation (11, 13). In line with this, mouse studies by Junxuan Lu's group show that methylseleninic acid, but not selenomethione, suppresses carcinogenesis in two mouse models of prostate cancer (14). In addition to forms of Se being critical for Se chemoprevention, one should take the U-shaped response into consideration, as additional Se intake benefit chemoprevention on those with low body Se status prior to entering the clinical trials (10).

Se metabolism is influenced by a variety of factors, such as individual genetic variation, smoking, alcoholism, age, disease and sex (15). Se metabolism, as well as the associations between Se status and cardiovascular disease, are sexually dimorphic (16, 17). Se is essential for male reproduction, in which a diet deprived of Se can affect testis development and spermiogenesis (18). The Se-dependent reduction of cancer risk, subfertility, or mortality in sepsis is mainly observed in males but not in females (17). Therefore, Se metabolism and health effects differ by sex, and generalizations should not be made across both sexes.

1.1.2 Selenoproteins

Se exerts its biological functions mainly through selenoproteins (2). Selenoproteins are a group of proteins that contains Sec, the 21st amino acid. Through genome sequencing and computational analysis, a total of 24 selenoproteins has been identified in rodents and 25 in humans (19). Gpx6 is the one found as selenoprotein in humans but not rodents (19). Compared with other metalloproteins, selenoproteins are unique. Instead of acting as a co-factor or prosthetic group, Se is specifically incorporated as Sec during selenoprotein translation.

Interestingly, the Sec acquired from foods cannot be utilized for selenoprotein synthesis directly. It needs to be synthesized *in vivo*. The initial step of Sec biosynthesis involves selenophosphate synthetase, which catalyzes the generation of selenophosphate using Se^{2-} and ATP (17). Meanwhile, a unique tRNA, designated $tRNA^{[Ser]Sec}$ is also needed for the synthesis of Sec (20).The $tRNA^{[Ser]Sec}$ is first aminoacylated with serine. The hydroxyl moiety of serine is then replaced by a phosphate group to form *O*-

phosphoseryl-tRNA$^{[Ser]Sec}$. Sec synthase replaces the phosphate group with activated selenophosphate to form selenocysteyl-tRNA$^{[Ser]Sec}$ (2).

The biosynthesis and specific incorporation of Sec into selenoproteins require a UGA codon in the coding region of selenoprotein mRNAs. UGA is generally a stop codon, but in the presence of a *cis*-acting sequence, a secondary structure within the 3'-UTR of selenoprotein mRNA, which is designated the Sec insertion sequence (SECIS) element, together with a couple of *trans*-acting factors, the UGA codon can be decoded as Sec (21). Besides the aforementioned selenocysteyl-tRNA$^{[Ser]Sec}$, the *trans*-acting factors also includes SECIS-binding protein 2 (SBP2) and Sec-specific elongation factor (eEFSec) (21). SBP2 possesses Sec incorporation and RNA-binding domains that are sufficient for three of the known functions of SBP2, including SECIS binding, ribosome binding, and *in vitro* Sec incorporation (21). eEFSec is the exclusive carrier of the selenocysteyl-tRNA$^{[Ser]Sec}$ (22).

There are also factors that selectively regulate the translation of some selenoproteins, instead of universally regulating them all. For example, nucleolin and eukaryotic initiation factor 4a3 have been found to bind to only a subset of selenoprotein mRNAs and regulate their translation (23, 24).

Most selenoproteins contain only one Sec in their C- or N-terminus. Selenoprotein P (SELP) is an exception. It contains up to 10 UGA codons in its coding region. Considering its Se transport function, the capacity of carrying multiple Sec seems needed (22).

Without sufficient Se supply, the synthesis of selenoproteins ends up with two possible consequences. Firstly, the UGA codon is decoded as a stop codon, and the

prematurely terminated selenoproteins are thus generated. Secondly, cysteine may replace Sec for the protein generation. The second processing method has only been identified in certain selenoproteins, such as TrxR1, thioredoxin reductase 2 (TrxR3), and SELP (25, 26).

Besides protein synthesis, mRNA expression of selenoproteins is also regulated by Se status (27). Certain selenoprotein mRNAs are very sensitive to Se deficiency, including *Gpx1*, selenoprotein H (*Selh*), and selenoprotein W (*Selw*) (28, 29). Nonsense mediated decay (NMD) has been shown to selectively degrade the mRNA of certain selenoproteins when Se is insufficient, such as *GPX1*, *SELW*, and *SELP* (30).

Selenoproteins have various physiological functions, from cellular redox regulation, thyroid hormone-activating to selenoproteins synthesis and Se transport (22). Selenoproteins have critical roles in both the glutathione (GSH)-dependent and Txn-dependent antioxidant systems (31). GPXs break down hydrogen peroxide (H_2O_2) and phospholipid peroxides into water. TrxRs catalyze the reduction of oxidized Txn (31).

Some selenoproteins have been directly linked to certain diseases. For instance, mutation of selenoprotein N (SEPN1) has been shown to cause a congenital muscular dystrophy with spinal rigidity and restrictive respiratory syndrome (32). In addition to selenoproteins, mutations in genes coding for proteins essential for selenoprotein expression such as SBP2 also show adverse phenotypes in humans (33, 34).

Transcription of selenoproteins is also sex dimorphic, and sex steroid hormones to some extent control selenoprotein transcription (17). The effects are not only exerted at the transcription level but also involve post-transcriptional modifications that cause tissue-specific expression patterns in males and females which vary with the Se status

(17). For instance, female mice have higher deiodinase 1 (*Dio1*) mRNA expression in liver. However, hepatic Dio1 activity is higher in male than in female mice (16).

1.2 Selenoprotein H

Different from the selenoproteins identified prior to the completion of the Human Genome Project in 2003, SELH was identified in mammals through selenoproteome screening by taking advantage of the features of selenoprotein genes to search for the entire genome (19). SELH widely exists crosses species. It has been first found in mammals such as *Homo sapiens* and *Mus musculus* (19). Almost at the same time, its homologous in *Drosophila melanogaster* was reported (35). *D. melanogaster* embryos with suppressed SELH expression showed decreased viability, development and antioxidant status (35). SELH has also been found in plants, but not in prokaryotes (22, 36).

SELH is a nucleolar protein with small molecular mass round 14 kDa (37). It is composed of a total of 122 amino acids, and the Sec is positioned at its 44^{th} amino acid (19). Compared to many other selenoproteins, SELH mRNA is very sensitive to dietary Se insufficiency (28, 29, 38). SELH mRNA level is regulated by Se status, senescence, and oxidative stress (39, 40). Interestingly, the expression of SELH mRNA is also under the regulation of metal transcription factor-1, which binds to the metal response element in the transcribed region of SELH (41).

How SELH protein product responds to depletion of Se is not unclear. SELH protein contains the conserved CXXU (C, Cys; X, any amino acid; U, Sec) motif, which may be a reversible thiolselenol/selenylsulfide redox group (37). Therefore, it belongs to the Txn-like family, along with selenoprotein T (SELT), selenoprotein V (SELV), and

SELW (42). The functions of SELH has been studied through knockdown and overexpression using cell models (37, 40, 43). The known functions of SELH include DNA-binding, redox regulation, and cellular senescence suppression (37, 40, 43). Lately, a study in zebrafish also discovered that *Selh*-deficient adults are prone to chemically-induced carcinogenesis, and p53 is a critical mediator of *Selh* (44).

Tissue expression pattern analysis by Western blot showed that spleen expresses SELH the greatest, followed by the brain (37). Neuronal protection by SELH has been reported (45-47). Overexpression of human SELH protects murine neuronal cells against UVB-induced cell death by reducing superoxide formation and inhibiting mitochondrial-initiated apoptosis (45, 46). Conversely, overexpression of SELH in neuronal cells promotes mitochondrial biogenesis and improves certain mitochondrial functions (47).

1.3 Aging-related Degeneration

1.3.1 Aging and cellular senescence

Aging is usually defined as the progressive loss of function accompanied by decreasing fertility and increasing mortality with advancing age (48). There are more than 100 theories of aging being proposed, the most studied of which include evolutionary, gene regulation, cellular senescence, free radical, and neuro-endocrine immune theories (49). It is putative that a gradual loss of cellular homeostasis acts as a driver of the aging process (50). Aging and longevity are controlled by multiple molecular and cellular signaling events that interface with environmental factors. Modulation of these pathways through gene mutations have been shown to extend lifespan (50). Food and nutrition also play important roles in extending lifespan or delaying cellular senescence (51).

Cellular senescence, on the other hand, refers to the phenomenon that a proliferation-competent cell undergoes permanent growth arrest (52). The senescent cell is characterized by a failure to re-enter the cell division cycle in response to mitogenic stimulation. Meanwhile, it also acquires resistance to oncogenic challenge (53).The causes of cellular senescence can be categorized into that induced by replication or stress. Replicative senescence usually happens in primary cells, which is caused by the shortening of telomeres after each round of cell division (54). The other form is stress-induced senescence attributed to DNA damage or mitogenic signals (53).

1.3.2 Telomere in senescence and aging

Telomeres are nucleoprotein complexes composed of repetitive DNA sequences (TTAGGG repeats in vertebrates) and an array of specialized proteins (54). They are located at the very ends of each linear chromosome and protect the chromosome ends from unscheduled DNA repair and degradation (55). Telomeric DNAs shorten after each round of DNA replication such that their length relates to the number of past replications and they could be considered biological markers of aging in normal human cells (55). Thus, telomere attrition has been implicated as a major mechanism of replicative senescence (54).

Dysfunctional telomere may happen when telomeric DNA shortens to a point below a threshold length, or the telomere-binding proteins are disrupted. Loss of telomeric protection can lead to end-to-end chromosome fusions, cell cycle arrest and/or apoptosis (54). In respond to that, cells may use two methods to extend their shortened telomere. All cancer cells activate either of these two methods, telomerase or alternate lengthening of telomere (ALT), to overcome replicative senescence (55). Telomerase is a

ribonucleoprotein complex composed of RNA and protein components. It can elongate the telomeric DNA sequence by binding to the open end of the telomeric DNA, using its RNA component, telomere RNA template (*TERC*), and its protein component, telomerase reverse transcriptase (TERT), to synthesis telomeric DNA sequence (54). Another major protein component of telomerase is dyskerin, which binds to both TERT and TERC and increases the stability of the complex (55). Significant telomerase activity is a feature of pluripotent stem cells and early stages of embryonic development. In contrast, telomerase activity in adult tissues is not sufficient to prevent telomere shortening in association with aging (55). Telomerase is activated in 80 – 90% of human carcinomas (56). The cancer cells use telomerase to overcome the telomere shortening, achieving the unlimited cell proliferation. Specialized immortal cells such as stem cells, germ cells, and T lymphocytes also have highly active telomerase and thus either maintain telomere length or delay telomere attrition (56). Another method of telomere elongation in mammals and yeast, ALT, depends on homologous recombination and occurs in 10 - 15 % of cancer cells (57).

Compared to humans, mice have longer telomere (40-80 vs. 10-15 kb), which may resist them to replicative senescence (58, 59). In 1997, the successful generation of telomerase-deficient mice by knocking out *Terc* was reported (58). The telomeres of the $Terc^{-/-}$ mice shorten at a rate of approximately 5 kb in every subsequent generation of $Terc^{-/-} \times Terc^{-/-}$ mating. $Terc^{-/-}$ mice show premature aging pathologies with an onset that is anticipated with increasing mouse generations (58). The anti-aging activity of telomerase has also been demonstrated by forcing telomerase expression in mice which showed augmented cancer resistance (55).

1.3.3 Se in aging-related degeneration

Multiple tissues undergo age-related degenerative changes, which in turn affects the function of these tissues (60-62). Tissue degenerative diseases are characterized by massive cell loss, ultimately leading to deterioration in quality or function of tissues and possible failure of vital organs (63). Examples of age-related degenerative diseases include Alzheimer's disease, cancer, type-2 diabetes, and heart disease (60, 62, 64).

Studies with model organisms suggest that aging and age-related conditions are manipulable processes that can be modified by both genetic and environmental factors, including dietary habits (51). Deficiency in Se can induce replicative senescence and shorten telomere length in primary cells (39). Our previous study also shows that, in *Terc*$^{-/-}$ mice, long-term dietary Se deprivation diminishes healthspan (65). The categorization of selenoproteins into essential and nonessential groups by McCann and Ames also suggests the aging-related function of certain selenoproteins (66). The functionality of specific selenoproteins in aging has been suggested, including SELH (40). The associations between Se or selenoproteins and age-related degenerative diseases are starting to be revealed. For instance, both deficiency of and excess in dietary Se can increase the risk of type-2 diabetes, presenting a U-shaped curve (67). The association between neurodegenerative diseases, such as Alzheimer's disease, and multiple selenoproteins, such as selenoprotein M (SELM) and SELP have also been suggested (68, 69).

CHAPTER II

EFFECTS OF LIFE-LONG DIETARY SELENIUM DEFICIENCY, AGING, AND SEX ON SELENOPROTEIN PROTEIN PRODUCTION AND SELENOTRANSCRIPTOME OF ELDERLY *TERC*$^{-/-}$ MOUSE

2.1 Abstract

The effects of dietary Se deficiency on life-span and health-span in aged short telomere mice have been investigated previously. The aim of this study was to study the effects of life-long dietary Se deficiency, aging, and sex on selenotranscriptome and identify the candidate selenoproteins that may play a role in mediating life-span and health-span in aged short telomere mice. In this study, using real-time PCR, mRNA expressions of all 24 selenoproteins in various tissues from male and female *Terc*$^{-/-}$ mice at 18 months or 24 months old by feeding Se deficiency diet or diet supplemented with Se from weanling were measured. Protein expression of selective selenoproteins and body Se status were also tested. In male mice, liver had more selenoprotein mRNAs changed by Se deficiency compared with kidney and heart. In females, kidney and liver had more numbers of selenorprotein mRNAs changed by Se deficiency than heart. A few selenoprotein mRNAs, *Gpx1*, *Selh*, and *Selw*, decreased by dietary Se deficiency cross all the tissues tested except testis, while aging did show such an effect on all tested selenoprotein mRNAs. Aging changed one selenoprotein mRNAs in male heart and 6 in

female heart, 6 in male kidney and 7 in female kidney, 13 in male liver but none in female liver, and one in testis. Further analysis show at least four unique patterns of selenotranscriptomic responses in a tissue-specific manner. First, there are sexually dimorphic responses of selenotranscriptome to dietary Se deprivation and aging. Second, a small group of selenoproteins responds to both Se deprivation and aging in parallel within a tissue. Third, there are selenoproteins up-regulated by aging or dietary Se deprivation in kidney. Fourth, certain selenoproteins, especially those in testis, are upregulated by aging in mice on a Se deficient diet. The effects of aging and life-long dietary Se deficiency have impacts on selenotranscriptome in a sex- and tissue-specific manner. The role Se playing in life-span and health-span is likely to act through combined effects on multiple selenoproteins. Considering the unparalleled changes of mRNA and protein levels of some selenoproteins, further studies on selenoproteins of interest at translational level are needed.

2.2 Introduction

The unique genomic features of in-frame UGA codon and SECIS element enable bioinformatics identification of selenoproteomes comprised of 24 selenoproteins in mice, as shown in Table 2.1 (19). Se, an essential mineral, is incorporated as Sec into selenoproteins by decoding UGA at translational level (70). There are hierarchies of Se distribution to tissues and selenoprotein expression upon body Se fluctuations. In response to dietary Se deficiency, this element is rapidly depleted in liver and kidney but preferably acquired and maintained in the testis and brain (71-73). Similarly, a subset of selenoproteins, including *Gpx1*, *Selh*, and *Selw*, are dwindling faster than other selenoproteins that are vital to life under Se-deficient conditions (28). Selenoprotein

hierarchy also varies considerably by species, tissues, sexes, and pathological conditions such as aging (16, 19, 28, 39, 74-77). For example, there are sexually dimorphic expression of Gpx1, iodothyronine deiodinase-1 (Dio1), and selenoprotein P (Sepp1) (74) and testis-specific expression of selenoprotein V (Selv) and the nuclear isoform of Gpx4 (19, 75). Furthermore, kidney and liver Se contents and TrxR activity are elevated in one-year-old compared with 35-day-old mice (76), and results of a 9-year longitudinal study on an elderly population show an age-dependent decline in plasma Se concentration (77).

Mammalian telomeres are composed of TTAGGG tandem repeats and associated proteins, which cap the ends of linear chromosomes (78). Due to the end-of-replication problem, telomeres in most proliferating cells shorten after each round of cell division. Telomere attrition eventually exposes chromosome ends, resulting in persistent DNA damage response and replicative senescence (79). Although mouse models have contributed tremendously to understanding human physiology and diseases, replicative senescence is resistant in mice due to their long telomeric DNAs in comparison to those in humans (25-80 vs. 10-15 kb) (58, 59). To circumvent this restriction, short telomere mice have been generated by breeding *Tert* knockout mice for a few generations to better model human aging and age-related disorders (58, 80, 81).

Se status progressively declines with age, according to a cohort of healthy subjects in Nove, Italy where no population migrates over the past 800 years (82). Patients with defective SBP2, an RNA-binding protein essential for the expression of all selenoproteins, display a multisystem disorder and shortened telomeres in mononuclear blood cells (34). Furthermore, Se or *SELH* deficiency accelerates replicative senescence in human primary fibroblasts (39, 40). While *Gpx1*$^{-/-}$ mice show no signs of aging except

cataract up to 20 months of age (83), primary *Gpx1*$^{-/-}$ embryonic fibroblasts exhibit senescence-like features (84). Our lab has recently shown that long-term dietary Se deprivation accelerates such aging phenotypes as grey hair, cataract, delayed wound healing, osteoporosis, and shortened telomeres in generation 3 (G3) *Terc*$^{-/-}$ mice carrying humanized telomere (65). In the present study, male and female G3 *Terc*$^{-/-}$ mice were fed a Se-deficient [Se(−)] or a Se-adequate [Se(+)] diet since weanling and their selenotranscriptomes in heart, kidney, liver, and testis were investigated at 18 and 24 months of age. GPX1 and GPX3 protein expression levels and Se concentrations in plasma and tissues were also measured.

2.3 Materials and Methods

2.3.1 Mice and diet

G3 *Terc*$^{-/-}$ short telomere mice under a C57BL/6 background (#004132, The Jackson Lab, Bar Harbor, ME) were used in this study. Weanling (3-wk-old) G3 *Terc*$^{-/-}$ mice were fed the modified AIN-93G basal diet [Se(−)] contained 30% torula yeast and 0.03 mg/kg Se by analysis (AOAC Method 986.15) or the Se(+) diet supplemented with 0.15 mg Se/kg as sodium selenate to the basal diet (#116053, #116063, Dyets, Inc. Bethlehem, PA) (85) until they were sacrificed at 18 or 24 months of age. Mice at 18-24 months of age are considered old (86). Mice were given free access to diets and distilled water, and housed in ventilated cages (up to 4 mice/cage) in an animal room (22°C, 12-h light:dark cycle). Our study was approved by the IACUC of the University of Maryland at College Park and Mississippi State University, and conducted in accordance with the NIH guides for the care and use of laboratory animals.

2.3.2 Sample collection

Mice were anesthetized with carbon dioxide and sacrificed by exsanguination via heart puncture using a heparinized syringe. Blood was allowed to clot and then centrifuged at 4°C (1,400 × *g* for 10 min). Plasma and organs were removed, snap-frozen in liquid nitrogen, and stored at -80°C for later analyses.

2.3.3 Selenotranscriptomic analyses by quantitative PCR (qPCR)

Liver, kidney, heart, and testis were homogenized in TRIzol and RNA was extracted following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA). Isolated RNA was quantified using an ND-2000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA (2 μg) was reversely transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Gene-specific primers targeting selenoprotein genes (Table 2.2) were designed by PrimerQuest Tool (Integrated DNA Technologies, Coralville, Iowa) for amplification of an approximate 100 bp segment of the cDNA through real-time PCR with SYBR Green Supermix (Bio-Rad Lab., Hercules, CA). Reactions were performed in an ABI Prism 7500 Fast Sequence Detection System (Thermo Fisher Scientific) with the following condition: 95°C for 30 sec, followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec. A dissociation curve was run for each plate to confirm the production of a single product. All of the 24 mouse Selenoprotein mRNAs were analyzed, but only those that showed statistically significant Se and/or aging effect using Bonferroni multiple comparison were shown. The β-actin mRNA was used as an internal reference. The actual values were calculated as relative to the amount of Se(+) mice at 18 months of age, which was set as 1.

2.3.4 Western blot and Se analyses

Tissues were broke down in RIPA buffer containing protease inhibitors (Santa Cruz Biotech., Santa Cruz, CA) by sonication. Homogenates were then centrifuged at 12,000 × *g* for 10 min at 4°C and the supernatant was collected. Proteins were separated by SDS-PAGE and then transferred to a PVDF membrane by a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (#1703940, Bio-Rad Lab.). Blots were incubated with primary antibodies (Gpx1, GTX116040, Genetex, Irvine, CA; Gpx3, AF4199, R&D Systems, Minneapolis, MN; β-actin, sc-8432, Santa Cruz Biotech.) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies (40). The Clarity Western ECL substrate (Bio-Rad Lab) was used for the image development and chemiluminescent signals were captured by Chemidoc-XS (Bio-Rad Lab.). Quantification of the band intensity was performed using the volume tool from Image Lab Software (Bio-Rad Lab.), and normalized with β-actin. Se contents in plasma and tissues were determined using a graphite furnace atomic absorption spectrophotometry with Zeeman-effect background correction (87) .

2.3.5 Statistical analyses

Results from homogeneous variance test showed that 11, 17, and 17 selenoprotein mRNAs in heart, liver, and kidney, respectively, had heterogeneous variances ($P < 0.05$) between male and female mice. Such unequal variance was confirmed by additional analyses of logarithmically transformed data. Thus, two-way ANOVA tests were conducted separately in males and females to determine the main effects of aging and dietary Se deficiency on selenotranscriptome. Pooled main effects or their interaction were shown for each selenoprotein. The Bonferroni multiple comparison was used to

determine the difference between each treatment group. Western blot analysis and Se concentration were analyzed by three-way ANOVA with aging, dietary Se deficiency and sex as the main treatments. The Pearson correlation coefficient method was used for the correlation analysis between plasma Se concentration and liver Se concentration. All analyses were conducted using SAS (SAS 9.4, Cary, NC). The level of significance (α) was set at 0.05 unless otherwise indicated.

2.4 Results

2.4.1 Effect of dietary Se deprivation on selenotranscriptomic changes

Long-term dietary Se deprivation decreased ($P \leq 0.05$) mRNA levels of 4 and 5 selenoproteins in heart, 4 and 16 in kidney, and 12 and 14 in liver of elderly males and females, respectively (Table 2.3, Figure 2.1 - 2.4), suggesting a tissue- and sex-specific effect on these selenotranscriptomic changes. Dietary Se deprivation consistently decreased mRNA levels of *Gpx1*, *Selh*, and *Selw* in heart, kidney, and liver of both sexes. While there were interactions between the main effects of Se and aging on male *Gpx1* and female *Selm* mRNAs in kidney (Table 2.2), their levels remained significantly decreased ($P < 0.05$) by dietary Se deprivation at both 18 and 24 months of age (Figure 2.3). In testis (Table 2.2, Figure 2.5), dietary Se deprivation had no statistically significant impact on the selenotranscriptome, although there was a trend of reduced (P = 0.09-0.11) mRNA levels of *Gpx4, Txnrd3*, and *Selv* in mice at 18 months of age.

Interestingly, dietary Se deprivation increased ($P < 0.05$) mRNA levels of *Dio2* and *Sepn1* in kidney of male mice (Table 2.3, Figure 2.3), *Txnrd1* in heart of 24-month males (Figure 2.2), and *Dio2* in heart of 24-month females (Figure 2.2). *Selv* was not detectable in heart, kidney or liver, nor were *Dio1* in heart of females and *Dio2* and *Dio3*

in liver of males. Besides *Gpx1* and *Selm* in kidney, there were interactions ($P \leq 0.05$) between the main effects of dietary Se deprivation and aging on mRNA levels of *Txnrd1* and *Dio2* in heart, *Selk*, *Dio1*, *Dio2*, and *Txnrd2* in liver, and *Selk* and selenoprotein S (*Sels*) in testis in a sex-specific manner (Table 2.2).

2.4.2 Effect of aging on selenotranscriptomic changes

Compared to 18 months, mice at 24 months of age had decreased ($P \leq 0.05$) mRNA levels of 1 and 6 selenoproteins in heart and 5 and 5 in kidney of males and females, respectively (Table 2.3, Figures 2.2 – 2.3). In liver, while aging resulted in decreased ($P \leq 0.05$) mRNA levels of 13 selenoproteins in males, there was none in females (Table 2.3, Figure 2.4). These decreases included *Selh* in heart of both sexes and liver of males; *Selo* in heart of females and kidney of both sexes; selenophosphate synthetase 2 (*Sephs2*) in kidney and liver of males and heart of females; methionine sulfoxide reductase B1 (*MsrB1*) and *Selw* in kidney of females and liver of males; *MsrB1* in heart of females; *Gpx4, Selm* and *Sels* in kidney and liver of males; *Txnrd3* in heart of females and liver of males; and *Selt* in heart of females and liver of males. In particular, mRNA levels of *Gpx1* and *Gpx3* were decreased only in kidney of females and *Selk*, *Txnrd1*, and *Txnrd2* only in liver of males. Furthermore, there were cases of decreased selenoprotein mRNA levels by aging present only in Se(−) or Se(+) mice. These included *Txnrd1* and *Selw* in heart of Se(+) males, *Gpx1* in kidney of Se(+) males, *Selm* in kidney of Se(−) females, and *Dio2* in liver of Se(+) females.

Another pattern was the elevated selenoprotein mRNA levels as the mice age. These increases included *Sepn1* in kidney of males; *Selk* and selenoprotein I (*Seli*) in

kidney of females; and *Sel15* in testis (Table 2.3). Furthermore, there were aging-related increases of *MsrB1* in heart of Se(−) males; *Dio2* in heart of Se(−) females; nine selenoproteins (*Gpx2*, *Gpx4*, *Sel15*, *Selk*, *Txnrd3*, *Sels*, *Sephs2*, *Selt* and *Selv)* in testes of Se(−) mice; *Gpx2* in heart of Se(+) females; and *Seli*, *Selk*, *Txnrd1*, and *Txnrd3* in liver of Se(+) females. No selenoprotein mRNA displayed an age-related induction in liver of male mice on a Se(−) or a Se(+) diet (Figures 2.2 – 2.5).

2.4.3 Selenoproteins mRNAs with parallel changes by dietary Se deprivation and aging

The mRNA levels of 11 selenoprotein genes were decreased by both dietary Se deprivation and aging in the same tissue (Table 2.3, Figures 2.2 – 2.4). These included *Selh* in heart of both sexes and liver of males; *Selm* in kidney and liver of males; *MsrB1* and *Selw* in kidney of females and liver of males; *Gpx1*, *Gpx3* and *Selo* in kidney of females; *Sel15*, *Txnrd1*, *Txnrd2*, and *Sels* in liver of males. In particular, *Selh* in heart was the only selenoprotein with such parallel decreases in both sexes. By contrast, mRNA levels of *Sepn1* in kidney of males were increased by both dietary Se deprivation and aging (Table 2.3, Figure 2.3).

2.4.4 Se concentration and selenoprotein expression at the protein level

Dietary Se deprivation significantly decreased ($P < 0.05$) plasma and liver Se concentrations in mice of both sexes at 18 and 24 months of age (Figure 2.6). Plasma Se concentration was greater (by 33%, $P < 0.05$) at 24 than 18 months of age in Se(+) males (Figure 2.6 A). In 18-month mice on a Se(+) diet, plasma Se concentration was greater (by 23%, $P < 0.05$) in females than males whereas liver Se concentration was greater (by 16%, $P < 0.05$) in males than females (Figure 2.6). Plasma and liver Se concentrations in

Se(−) mice did not differ by age or sex. Results of regression analyses demonstrated that Se concentrations in plasma and liver were positively associated ($P < 0.05$) in Se(−) females ($\gamma = 0.86$), Se(−) males ($\gamma = 0.55$), and Se(+) females ($\gamma = 0.63$), but not in Se(+) males ($\gamma = 0.22$, $P = 0.55$).

Increasing lines of recent evidence support the notion that selenoprotein mRNA and protein expression do not always go in a parallel manner (39, 84, 88). Results from Western blot analyses showed that dietary Se deprivation decreased ($P < 0.05$) protein levels of GPX1 in heart, kidney, and liver (Figure 2.7A) and GPX3 in plasma and kidney (Figure 2.7 B) regardless of age or sex. GPX1 decreased in kidney and liver at both transcriptional and translational levels by Se deprivation, but the extent of reduction was much more remarkable at the latter than the former. GPX1 protein levels were not affected by age or sex in liver and kidney, but were decreased ($P < 0.05$) in heart of males at 24 than 18 months of age and of females than males at 18 months. The extent of GPX1 protein decrease by dietary Se deprivation was smaller in heart compared with that in liver and kidney. Dietary Se deprivation decreased GPX3 protein levels to a greater extent in plasma (91-93%) than kidney (52-68%) in males, but comparable between plasma (75-78%) and kidney (73-82%) in females. While GPX3 protein levels were not affected by age or sex in plasma or age in kidney, they were greater in kidney of females than males on a Se(+) diet. This may correlate with their mRNA expression changes.

2.5 Discussion

The effect of dietary Se deficiency (5-16 weeks) on selenotranscriptomic changes in mice, pigs, and chicks (28, 89-91) and the effect of sex and age (up to 1-year old) on

mRNA expression of four mouse selenoproteins (28, 76), *Gpx1*, *Gpx3*, *Dio1*, and *Sepp1*, have been studied mainly in young animals. Herein, short telomere mice that model human aging better (58, 80, 81) were employed to comprehensively determine the impact of long-term dietary Se deprivation, sex, and age on selenotranscriptomes and body Se status in tissues of late-life mice. Adding to the well-studied topic of Se regulation on selenotranscriptomes (92), here new insights into selenotranscriptomic regulation by aging and sex in mice with humanized telomeres were provided. The results show at least four unique patterns of selenotranscriptomic responses in a tissue-specific manner. First, there are sexually dimorphic responses of selenotranscriptome to dietary Se deprivation and aging. Second, a small group of selenoproteins responds to both Se deprivation and aging in parallel within a tissue. Third, there are selenoproteins up-regulated by aging or dietary Se deprivation in the kidney. Fourth, certain selenoproteins, especially those in the testis, are upregulated by aging in mice on a Se(−) diet.

While it is inconclusive whether there is survival advantage of one sex over the other based on analyses of 118 publications, results from studies that standardize environmental conditions show a 13% increase in median longevity in female than male mice (93). Consistent with this notion, previously our lab found that females outlive males in the mice and dietary Se deprivation is predicted to extend lifespan to a greater extent in females than males (65). It is thus intuitive to conjecture whether sexually dimorphic responses of selenoprotein to dietary Se deprivation and aging are linked to longevity. The results show that aging clearly down-regulates the selenotranscriptomes in sex-specific manner; however, quantitative and profiling changes of selenoproteins in males and females diverge in heart, kidney, and liver. Similarly, sexual dimorphic

expression of *Sepp1* and *Dio1*, but not *Gpx1* or *Gpx3*, mRNAs have been shown in liver and kidney of young mice on a C57BL/6 background (16). The results show that aging down-regulates 13 selenoproteins in liver of males but none in females, whereas the opposite trend is true in heart. Among the selenoproteins down-regulated by aging in kidney, *Selo* mRNA is affected in both sexes. In comparison, dietary Se deprivation impacts the sexually dimorphic pattern of selenotranscriptomes greatly in kidney, but to a less extent in heart and liver. Although it is immature to implicate specific or a combination of sexually dimorphic selenoproteins in longevity advantage in the female mice, these selenoprotein profiles in various tissues are valuable for future informatics analyses of longevity when physiological functions of selenoproteomes are better understood. Cautious consideration, however, should be taken as levels of selenoprotein transcripts are not necessarily consistent with those of proteins (88, 89). Lifespan in males is extended by castration in eunuchs (94, 95) and by supplementation with 17α-estradiol, a stereoisomer of the predominant female hormone 17β-estradiol and an inhibitor of testosterone activation, in mice (96). Furthermore, orchiectomy but not ovariectomy results in reduced *Sepp1* and *Gpx3* mRNA levels in kidney of mice (16), and orchiectomy increases brain Se levels and attenuates neurodegeneration phenotypes in *Scly*$^{-/-}$*Sepp1*$^{-/-}$ mice with compromised Se transport and release (71-73). Therefore, another line of interesting research is to determine whether sex hormones confer their impact on healthspan and lifespan through selenoproteins.

Second, one prime goal of this study is to relate candidate selenoproteins to premature aging-like phenotypes in Se(−) short telomere mice. Based on the triage theory of aging by McCann and Ames, low hierarchy selenoproteins that are non-essential and

sensitive to modest Se deficiency may contribute to age-associated diseases (66). Although selenotranscriptomes are diversely changed by dietary Se deprivation and aging in both sexes, there are 11 selenoproteins that are down-regulated by dietary Se deprivation and also by aging in Se(+) mice in heart, kidney, and/or liver. They can be functionally categorized to thioredoxin reductases (*Txnrd1* and *Txnrd2*), the Rnx-like family with a CXXU motif (*Selh*, *Selm*, *Selw*, and *Selo*), glutathione peroxidases (*Gpx1* and *Gpx3*), and those capable of protein quality control or repair (*Sel15*, *MsrB1*, and *Sels*). Known functions of these selenoproteins (22) support the notion that Se at nutritional levels of intake may delay age-related degenerations through redox maintenance and protein quality control.

Third, kidney *Sepn1* mRNA is up-regulated in males and *Selk* and *Seli* in females by aging. One common feature of these three selenoproteins is their cellular localization in endoplasmic reticulum (22). As endoplasmic reticulum stress may contribute to aging (97), it is tempting to speculate that up-regulation of these selenoproteins by aging is a compensatory response for maintenance of protein homeostasis and optimal aging. In particular, Sepn1 is known to maintain redox-related calcium homeostatis (98) and protects against muscle degenerations and the associated kyphosis (99-101). Furthermore, kidney *Dio2* and *Sepn1* mRNA levels are up-regulated by dietary Se deprivation in males. One plausible explanation of such responses to dietary Se(−) conditions is the regulation by redox-sensitive activators of transcription such as NF-κB, as the human *DIO2* promoter contains NF-κB responsive element (102) and Se deficiency can activate and translocate NF-κB to the nucleus (103). While it is unclear why mRNA levels of kidney *Sepn1* are elevated in the Se(−) mice, this trend is

consistently observed in skeletal muscles of chicks on a Se(−) diet (104). Dietary Se deficiency may also up-regulate selenoprotein expression through down-regulation of microRNAs that target selenoprotein mRNA (105). Whatever the mechanism of selenoprotein induction by Se deficiency, results from *Dio2*$^{-/-}$ mice suggest protective roles of Dio2 against hearing loss, cognition declines, and defective glucose metabolism (106-108) and Se(−) mice display type-2 diabetes-like symptoms (109). Endoplasmic reticulum selenoproteins and *Dio2* may be up-regulated to alleviate age-related degeneration upon normal aging or acceleration by Se deficiency.

Fourth, aging elevates selenoprotein mRNA levels in the testes of Se(−) mice such that no selenoprotein mRNA is down-regulated by dietary Se deficiency at 24 months of age. This pattern is absent in the other three tissues tested in this study. Although these nine selenoproteins that are ranked high in selenoprotein hierarchy in other tissues (28, 29), our results suggest that they may be dispensable at 18 months but essential at 24 months of age in testes. Of them, *Sephs2* shows such a trend also specific to testes. Deficiency in *Sephs2*, a key enzyme for the formation of selenocysteine-tRNA, stalls selenoprotein translation and subsequently induces selenoprotein mRNA degradation. Because not all the selenoprotein mRNAs sensitive to dietary Se deficiency in the testes are targets of NMD (30), other mechanisms such as regulation by selenocysteine-tRNA isoforms (110) and the SECIS-binding eIF4a3 and nucleolin (23, 24) may account for increased levels of selenoprotein mRNA by aging in Se(−) testes. Those regulatory factors have been summarized in Table 2.4. Such pattern of induction appears to be a compensatory response. Dietary Se deprivation induces oxidative stress, which, in turn, may transactivate the expression of certain selenoproteins such as *Gpx2*

through its antioxidant response element (111), *Sels* by NF-κB binding (112), and *Txnrd3* by Klf9 binding (113). While exact functions of many of these proteins in testes remain unknown, Gpx4 plays a peroxidase-independent, structural role in mature spermatozoa (75) and Txnrd3 accumulates at relatively high levels in testes after puberty (114). The testes-specific Selv is thought to be evolutionarily duplicated from Selw, whose mRNA is most sensitive to NMD (19, 115). As many selenoproteins are known to carry multiple roles, such age-dependent induction of selenoproteins may enable the old mice to cope with various physiological conditions in late life.

Testis is ranked high in Se hierarchy for optimized male production (71, 116), as it expresses very high levels of apolipoprotein E receptor-2 that binds and enables SELP to preferentially deliver Se to this organ (117, 118). As such, mitochondrial Gpx4 in spermatocytes can maintain optimal sperm quality and fertility even under Se deficiency (119, 120). However, Se retention in testes may compromise healthspan, as castration alleviates the neurodegeneration phenotypes in *Sepp1*$^{-/-}$ mice (73). Thus, under conditions of Se deficiency, the body appears to favor production early in life at the expense of degenerative diseases later in life, supporting the triage theory of aging.

While the recommended daily allowance for Se is both 55 μg/day in adult males and females, human studies collectively suggest modestly higher serum Se concentrations in adult males than females (1.14 vs. 1.09 μM, $P < 0.0001$) (121). While serum, liver, and kidney Se concentrations are comparable between males and females in young rats (16), serum and liver Se concentrations are greater and lower, respectively, in female than male mice at 18 months of age. It appears that, compared to females, males sequester

related more Se in liver than in plasma, and Se is released from liver to plasma in late-life males.

Dietary Se deficiency deceases the tissue-specific expression of selenoprotein, of which *Gpx1*, *Selh*, and *Selw* mRNAs are ranked low in selenoprotein hierarchy in liver, kidney, and/or colon of young male mice (28, 29). Consistent to and extension of these results, they are most sensitive to dietary Se deprivation in the heart, kidney, and liver of elderly mice in both sexes at old age. In a previous study on piglets (89), the results show that selenotranscriptomic changes in heart by dietary Se-deficiency is less significant compared with liver and kidney. This is thought to be attributed to tissue Se hierarchy, as liver and kidney supply Se to other tissues and contain 1-2 fold greater Se than heart (122). A puzzling yet interesting question is: why the hierarchy of other selenoproteins differ by Se status, tissue, sex, and age? For example, the testis-specific decline in *Gpx4* mRNA level by long-term dietary Se deprivation cannot be explained by NMD, as *Gpx4* mRNA is resistance to this degradation pathway (30). It is thus of future interests to study whether the efficacy of different pathways regulating selenoprotein mRNA levels varies by tissue, and whether they differ by age and sex.

2.6 Figures and Tables

Table 2.1 The mouse selenoproteome

Glutathione peroxidases (Gpxs) family
Gpx1
Gpx2
Gpx3
Gpx4
Thioredoxin reductases (Txnrds) family
Txnrd1
Txnrd2
Txnrd3
Iodothyronine deiodinases (Dios) family
Dio1
Dio2
Dio3
Selenoprotein 15 and M family
Sel15
Selm
Selenoprotein S and K family
Sels
Selk
Thioredoxin(Rxn)-like proteins family
Selw
Selh
Selt
Selv
Other selenoproteins
Sepp1
Sephs2
MsrB1
Seln
Seli
Selo

Table 2.2 Primers for qPCR analyses of selenotranscriptome

Gene	Forward	Reverse	Size (bp)
β-actin	GAGGTATCCTGACCCTGAAGTA	CACACGCAGCTCATTGTAGA	104
G*px1*	GGTTCGAGCCCAATTTTACA	CCCACCAGGAACTTCTCAAA	199
Gpx2	CTCAGTGTACCCTCGGGAGA	AAAGGAAATGGCCGGTAGGG	84
Gpx3	GATGTGAACGGGGAGAAAGA	CCCACCAGGAACTTCTCAAA	152
Gpx4	CTCCATGCACGAATTCTCAG	ACGTCAGTTTTGCCTCATTG	117
Dio1	GGAACCATAGGCATTGGAAA	AGTGCCAGAGAGCCAGATTC	152
Dio2	CACCGGCTCTATTCTTCATACC	GTTCAGACTCACCTTGGAACA	96
Dio3	CTCGAAACAGCGCCTAAAGTA	GAAGTCCATCCCTTACCATGTC	100
Txnrd1	TTCGACCTGATCATCATTGG	CCACATTCACACACGTTCCT	151
Txnrd2	CACAGGTGATGCAGACAGTAG	CTCAGCAACCAGTCACAGTAG	108
Txnrd3	CCTTTCCCAGTTGCTAGTGC	GTGCTACACTCTGGGCAACA	107
Sel15	CTCACCAGTGAAACGCTTTG	TCAAAGAGCACACAGCAAGG	143
Selm	TTTGTCACCGAGGACATTCA	TGTACCAGCGCATTGATCTC	155
Selh	GCGAGATTTGAACTTTGCATC	TTGTCCACCGTCTCCATAGG	162
Selt	TGTGGCAACAGAAAGGGATT	CAGGTGGCATCAACATCAAG	115
Selw	CCCAAGTACCTCCAGCTCAA	GCCATCACCTCTCTTCTTGG	147
Selk	GCTGGTGGATGAGGAAGGTA	CTCATTCATCTGTGGGGACA	158
Sels	GCCTTACGCACACTTTCACA	GTGGCCTAATGGCAATGTCT	119
Seli	GTCTGTTGCTTTGTGTGGATTT	CATCTACTGTGAGGGCTTTCTC	109
Sepp1	GCAATTGCTTGACAGTGTGC	TTCATGGGCTGATTTTGTCA	131
Selo	CTGGCTGAGGCCATTCTAAA	GTGTCCCATCTTCTTCCTTCTC	115
MsrB1	ACAGTTGTTGCCCCATTAGC	GGAGTGGGTCTCAGCTTCAG	154
Sepn1	TGTTGACCTGATGACCCAAG	CTAGACAGTGCTGGCAATAAGA	109
Sephs2	CCTGTTCTGGACAAGAGTTGAG	TTACTGGGAGTTAGAGGGAGAG	102
Selv	CTGGAGTTTGAGGAGGAAAGAG	TCGTCAATAGCACCCACAAG	143

Gpx1, glutathione peroxide 1; Gpx2, glutathione peroxidase 2; Gpx3, glutathione peroxidase 3; Gpx4, glutathione peroxidase 4; Dio1, iodothyronine deiodinase 1; Dio2, iodothyronine deiodinase 2; Dio3, iodothyronine deiodinase 3; Txnrd 1, thioredoxin reductase 1; Txnrd 2, thioredoxin reductase 2; Txnrd 3, thioredoxin reductase 3; Sel15, the 15 kDa selenoprotein; Selm, selenoprotein M; Selh, selenoprotein H; Selt, selenoprotein T; Selw, selenoprotein W; Selk, selenoprotein K; Sels, selenoprotein S; Seli, selenoprotein I; Sepp1, selenoprotein P; Selo, selenoprotein O; MsrB1, methionine sulfoxide reductase B1; Sepn1, selenoprotein N; Sephs2, Selenophosphate Synthetase 2; Selv, selenoprotein V

Table 2.3 A summary of selenoproteins displaying significant ($P \leq 0.05$) main effects of aging, dietary Se deprivation, and their interactions in the selenotranscriptome of mouse tissues determined by two-way ANOVA[123]

Tissue	Male			Female		
	Se(-)	Aging	Interaction	Se(-)	Aging	Interaction
Heart	*Selw* *Selh* *Gpx1* *Sel15*	*Selh*	*Txnrd1*	*Gpx2* *Selw* *Gpx1* *Selh* *Selm*	*Txnrd3* *Sephs2* *Selo* *Selt* *MsrB1* *Selh*	*Dio2*
Total	4	1	1	5	6	1
Kidney	*Gpx1*[&] *Selm* *Selw* *Selh* *Dio2* * *Sepn1* *	*Sephs2* *Selo* *Sels* *Gpx4* *Selm* *Sepn1* *	*Gpx1*	*Selw* *Selh* *Selm*[&] *Gpx1* *Sepp1* *Gpx3* *Sels* *Txnrd2* *Txnrd1* *Selo* *Selt* *MsrB1* *Selk* *Gpx4* *Sel15* *Seli*	*MsrB1* *Gpx3* *Gpx1* *Selw* *Selo* *Selk* * *Seli* *	*Selm*
Total	6	6	1	16	7	1
Liver	*Selw* *Gpx1* *Dio1* *Selh* *Gpx3* *Txnrd1*	*Txnrd2* *Sephs2* *Gpx4* *Selh* *Selt* *MsrB1*	*None*	*Selw* *Dio3* *Gpx3* *Txnrd1* *Selh* *Txnrd3*	*None*	*Dio1* *Dio2* *Txnrd2* *Selk*

Table 2.3 (Continued)

	Sels	*Txnrd3*		*Sepp1*		
	Seli	*Selm*		*Gpx1*		
	Txnrd2	*Sel15*		*Selm*		
	MsrB1	*Txnrd1*		*Gpx2*		
	Sel15	*Selk*		*MsrB1*		
	Selm	*Sels*		*Gpx4*		
		Selw		*Sepn1*		
				Sel15		
Total	12	13	0	14	0	4
Testis	*None*	*Sel15**	*Selk*			
			Sels			
Total	0	1	2			

[1]Selenoproteins are listed in the descending order of the extent of mRNA level decline by dietary Se deprivation or aging. In kidney, selenoproteins marked with an asterisk (*) show increased mRNA levels by dietary Se deprivation or aging. Se(−), dietary Se deprivation, and selenoproteins marked with an ampersand ([&]) show significant decreased mRNA level at both 18 and 24 months even the interaction between dietary Se deprivation and aging is significant.
[2]Selenoproteins shown in red color represent those display consistent mRNA changes by both dietary Se deprivation and aging in the tissue.
[3]Gpx1, glutathione peroxide 1; Gpx2, glutathione peroxidase 2; Gpx3, glutathione peroxidase 3; Gpx4, glutathione peroxidase 4; Dio1, iodothyronine deiodinase 1; Dio2, iodothyronine deiodinase 2; Dio3, iodothyronine deiodinase 3; Txnrd 1, thioredoxin reductase 1; Txnrd 2, thioredoxin reductase 2; Txnrd 3, thioredoxin reductase 3; Sel15, the 15 kDa selenoprotein; Selm, selenoprotein M; Selh, selenoprotein H; Selt, selenoprotein T; Selw, selenoprotein W; Selk, selenoprotein K; Sels, selenoprotein S; Seli, selenoprotein I; Sepp1, selenoprotein P; Selo, selenoprotein O; MsrB1, methionine sulfoxide reductase B1; Sepn1, selenoprotein N; Selv, selenoprotein V

Table 2.4 Sensitivity of selenoproteins to NMD, i^6A^- mutant tRNA $^{[Ser]Sec}$, and SECIS-binding factors (23, 24, 30, 110)

		Sensitive	Insensitive
NMD (human cell)		*Gpx1, Sepw1, Sepp1*	*Gpx4, Seli, Selo, MsrB1, Sephs2*
i^6A- mutant tRNA $^{[Ser]Sec}$ (mouse tissue)		*Gpx1, Gpx3, Gpx4* in liver, *Dio1, Dio2, Txnrd, Selt* in brain and kidney, *Sepp1*	*Gpx4* in testis, *Txnrd2*, *Selt* in testis
SECIS-binding factors (rat cells)	eIF4a3	*Gpx1, MsrB1*	*Gpx4, Txnrd1*
	Nucelolin binding	*Gpx2, Gpx4, Dio1, Dio2, Txnrd1, Sepw1, Selo, MsrB1, Seln*	*Gpx1, Sel15, Selt*

Gpx1, glutathione peroxide 1; Gpx2, glutathione peroxidase 2; Gpx3, glutathione peroxidase 3; Gpx4, glutathione peroxidase 4; Dio1, iodothyronine deiodinase 1; Dio2, iodothyronine deiodinase 2; Txnrd 1, thioredoxin reductase 1; Txnrd 2, thioredoxin reductase 2; Sel15, the 15 kDa selenoprotein; Selt, selenoprotein T; Selw, selenoprotein W; Seli, selenoprotein I; Sepp1, selenoprotein P; Selo, selenoprotein O; MsrB1, methionine sulfoxide reductase B1; Sephs2, selenophosphate synthetase 2

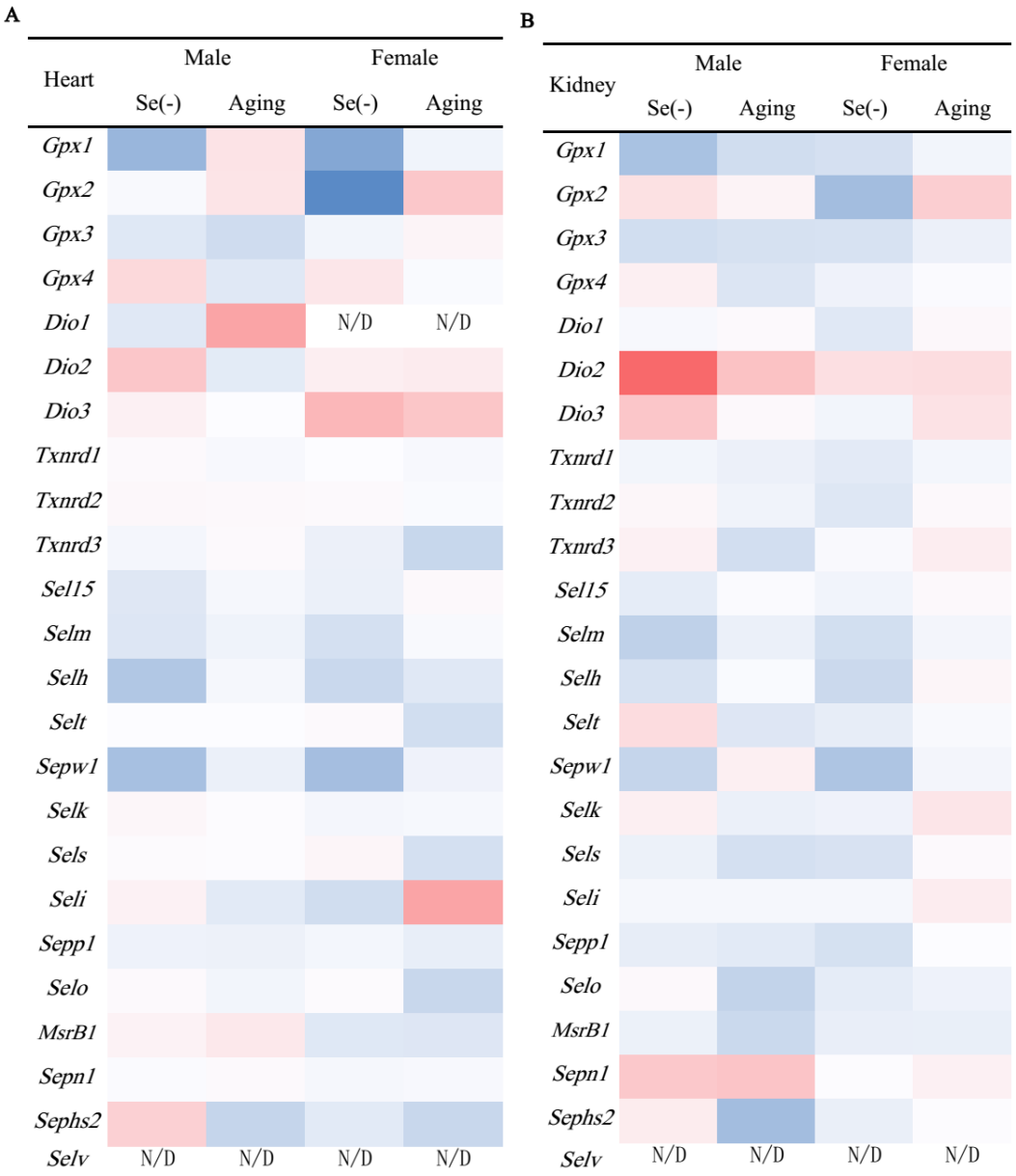

Figure 2.1 Visualization of effects of dietary Se and aging on selenoprotein mRNA expression

Relative change for each gene per tissue were calculated relative to 18 months Se-adequate expression and set to 1. Expression levels were visualized using Microsoft Excel, as shown in the color scale. Gpx1, glutathione peroxide 1; Gpx2, glutathione peroxidase 2; Gpx3, glutathione peroxidase 3; Gpx4, glutathione peroxidase 4; Dio1, iodothyronine deiodinase 1; Dio2, iodothyronine deiodinase 2; Dio3, iodothyronine deiodinase 3; Txnrd 1, thioredoxin reductase 1; Txnrd 2, thioredoxin reductase 2; Txnrd 3, thioredoxin reductase 3; Sel15, the 15 kDa selenoprotein; Selm, selenoprotein M; Selh, selenoprotein H; Selt, selenoprotein T; Selw, selenoprotein W; Selk, selenoprotein K; Sels, selenoprotein S; Seli, selenoprotein I; Sepp1, selenoprotein P; Selo, selenoprotein O; MsrB1, methionine sulfoxide reductase B1; Sepn1, selenoprotein N; Sephs2, Selenophosphate Synthetase 2; Selv, selenoprotein V; N/D, undetectable

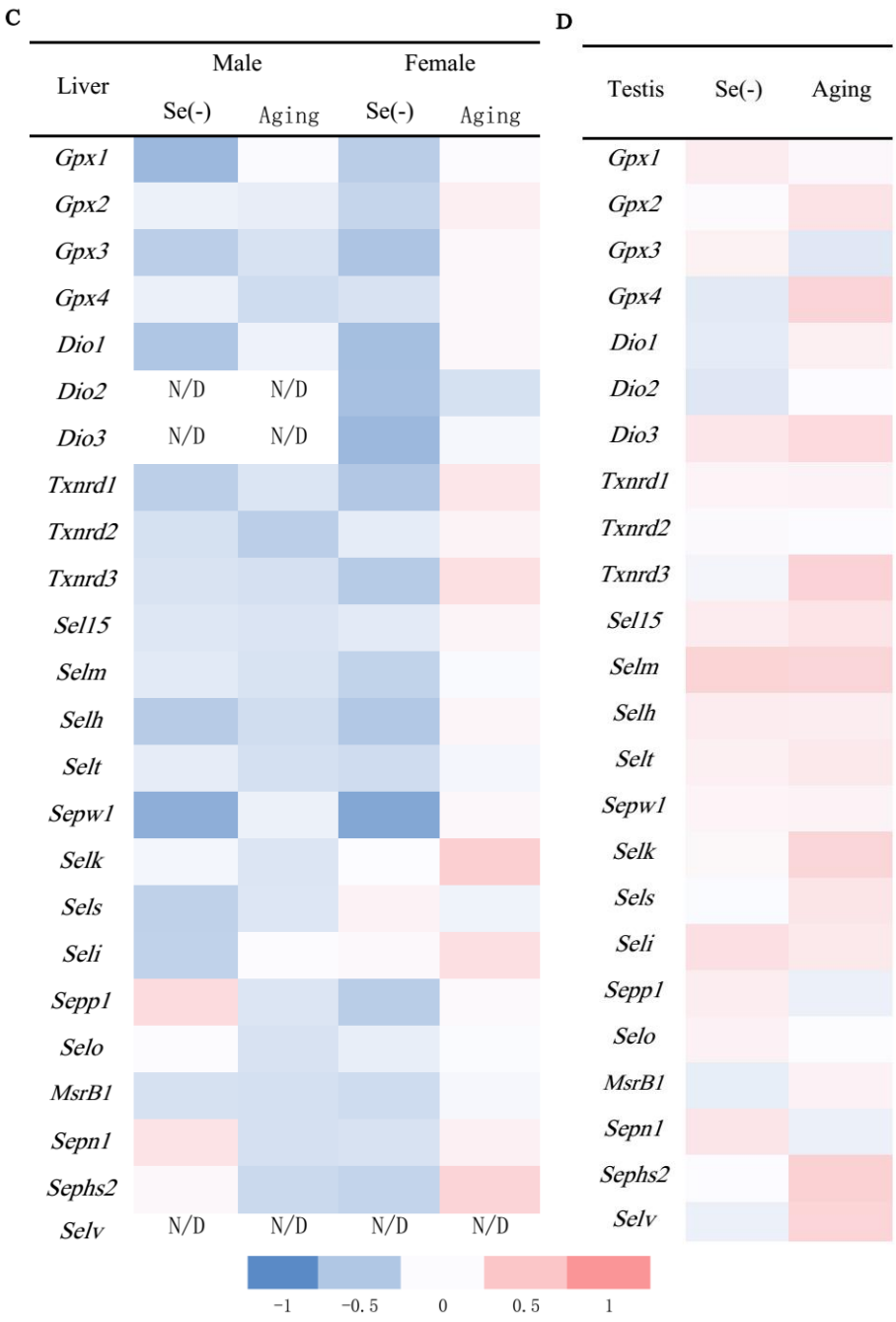

Figure 2.1 (Continued)

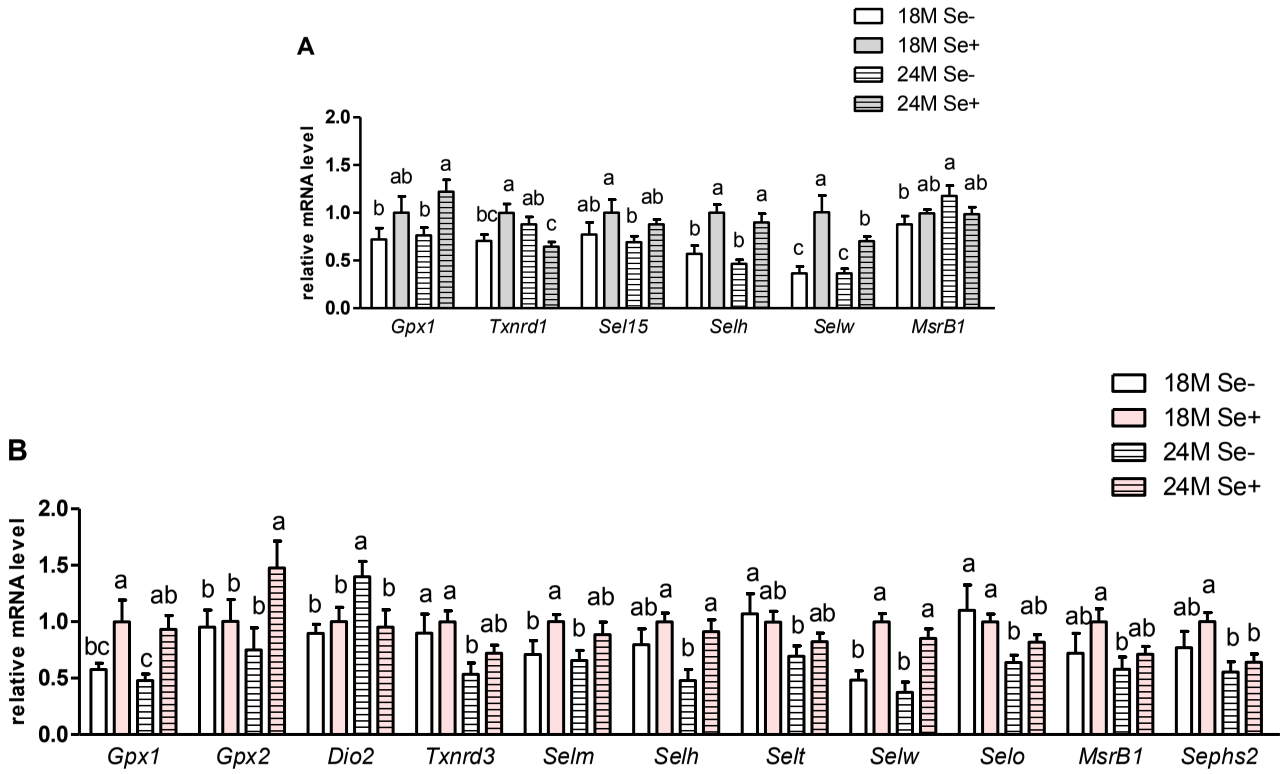

Figure 2.2 Effect of aging and dietary Se deprivation on (A) male heart and (B) female heart.

Selenotranscriptome in G3 *Terc*$^{-/-}$ mice determined by qPCR. Data are means ± SEM, n = 5-10. Within a selenoprotein, means without a common letter differ ($P < 0.05$). Selenoproteins are not shown if they do not have any treatment effect. Gpx1, glutathione peroxide 1; Gpx2, glutathione peroxidase 2; Dio2, iodothyronine deiodinase 2; Txnrd 1, thioredoxin reductase 1; Txnrd 3, thioredoxin reductase 3; Sel15, the 15 kDa selenoprotein; Selm, selenoprotein M; Selh, selenoprotein H; Selt, selenoprotein T; Selw, selenoprotein W; Selo, selenoprotein O; MsrB1, methionine sulfoxide reductase B1; Sephs2, selenophosphate synthetase 2.

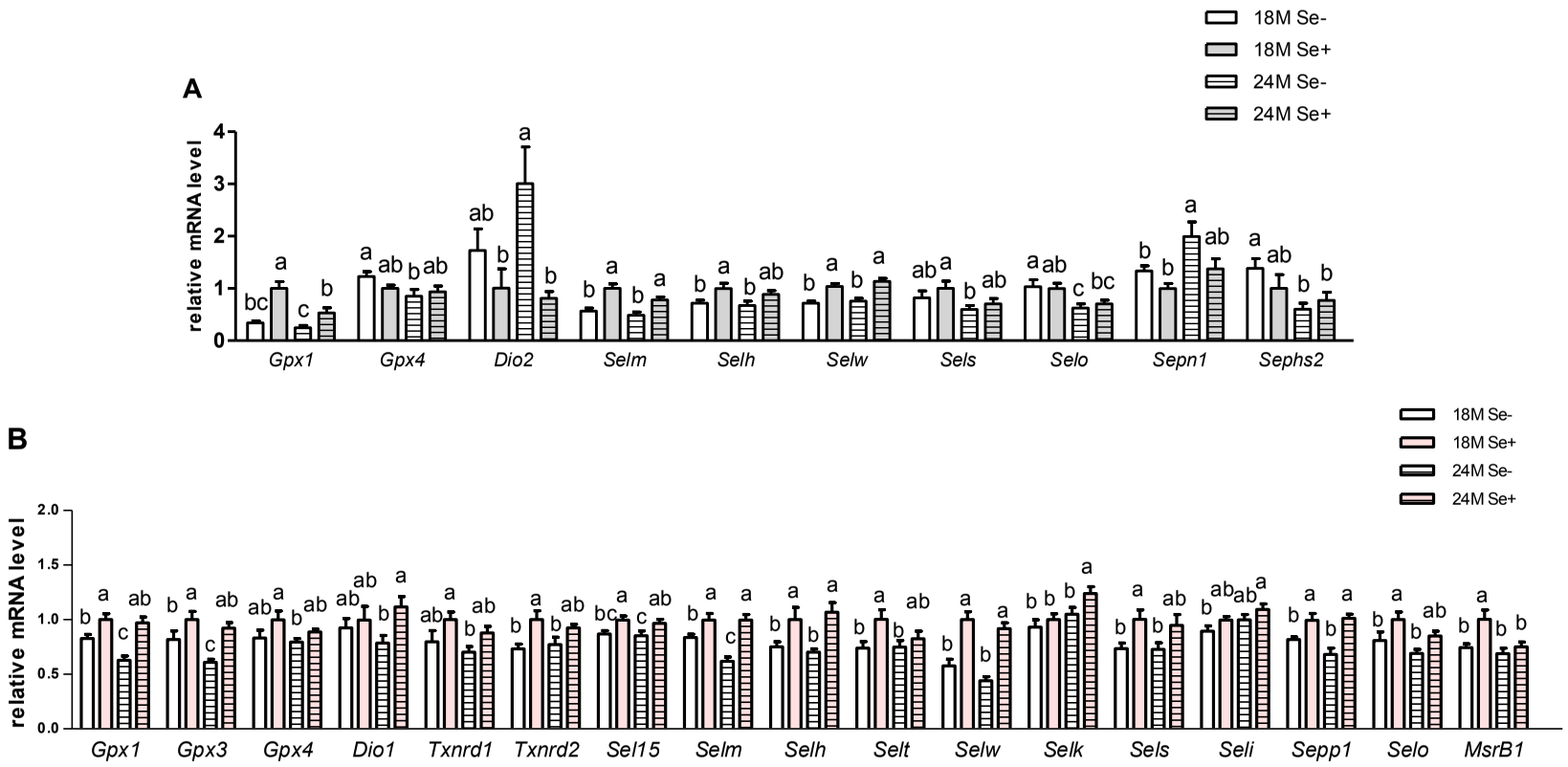

Figure 2.3 Effect of aging and dietary Se deprivation on (A) male kidney and (B) female kidney.

Selenotranscriptome in G3 *Terc*$^{-/-}$ mice determined by qPCR. Data are means ± SEM, n = 5-10. Within a selenoprotein, means without a common letter differ ($P < 0.05$). Selenoproteins are not shown if they do not have any treatment effect. Gpx1, glutathione peroxide 1; Gpx3, glutathione peroxidase 3; Gpx4, glutathione peroxidase 4; Dio1, iodothyronine deiodinase 1; Dio2, iodothyronine deiodinase 2; Txnrd 1, thioredoxin reductase 1; Txnrd 2, thioredoxin reductase 2; Sel15, the 15 kDa selenoprotein; Selm, selenoprotein M; Selh, selenoprotein H; Selt, selenoprotein T; Selw, selenoprotein W; Selk, selenoprotein K; Sels, selenoprotein S; Seli, selenoprotein I; Sepp1, selenoprotein P; Selo, selenoprotein O; MsrB1, methionine sulfoxide reductase B1; Sepn1, selenoprotein N

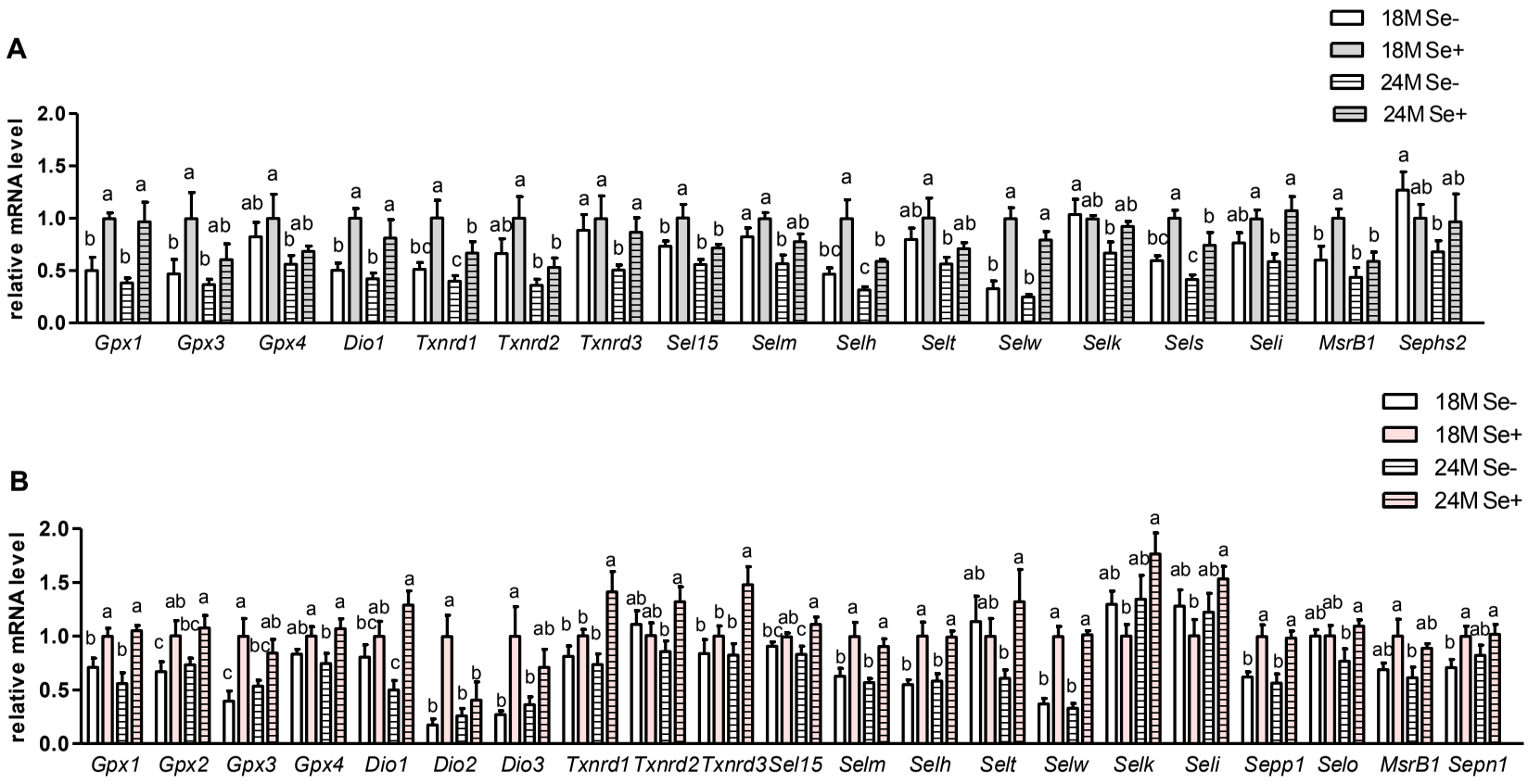

Figure 2.4 Effect of aging and dietary Se deprivation on (A) male liver and (B) female liver.

Selenotranscriptome in G3 *Terc*$^{-/-}$ mice determined by qPCR. Data are means ± SEM, n = 5-10. Within a selenoprotein, means without a common letter differ ($P < 0.05$). Selenoproteins are not shown if they do not have any treatment effect. Gpx1, glutathione peroxide 1; Gpx2, glutathione peroxidase 2; Gpx3, glutathione peroxidase 3; Gpx4, glutathione peroxidase 4; Dio1, iodothyronine deiodinase 1; Dio2, iodothyronine deiodinase 2; Dio3, iodothyronine deiodinase 3; Txnrd 1, thioredoxin reductase 1; Txnrd 2, thioredoxin reductase 2; Txnrd 3, thioredoxin reductase 3; Sel15, the 15 kDa selenoprotein; Selm, selenoprotein M; Selh, selenoprotein H; Selt, selenoprotein T; Selw, selenoprotein W; Selk, selenoprotein K; Sels, selenoprotein S; Seli, selenoprotein I; Sepp1, selenoprotein P; Selo, selenoprotein O; MsrB1, methionine sulfoxide reductase B1; Sepn1, selenoprotein N; Sephs2, selenophosphate synthetase 2

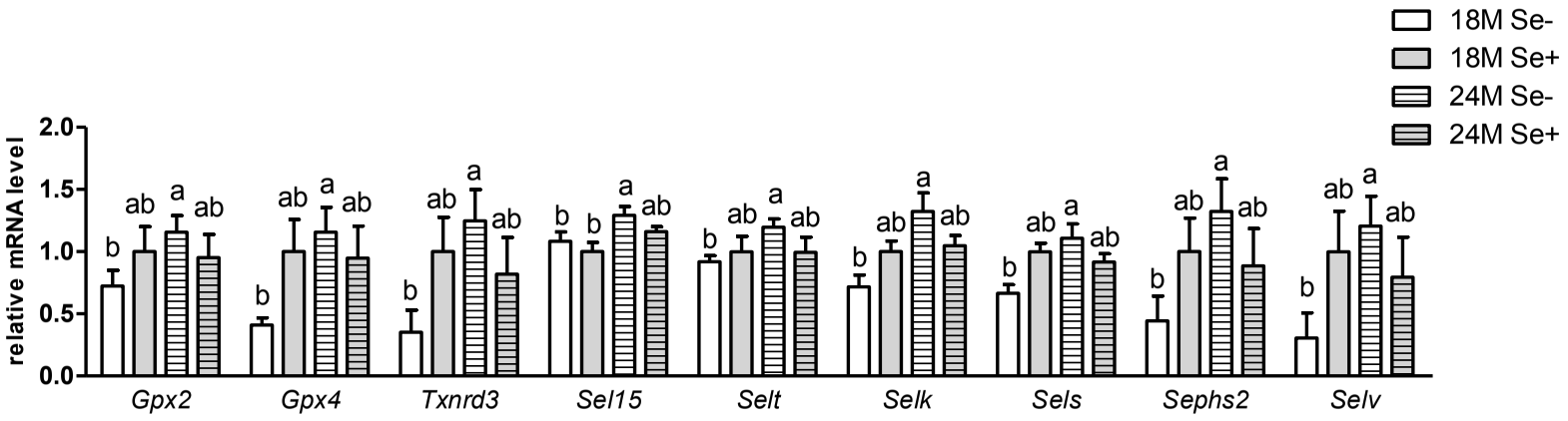

Figure 2.5 Effect of aging and dietary Se deprivation on testis.

Selenotranscriptome in G3 *Terc*$^{-/-}$ mice determined by qPCR. Data are means ± SEM, n = 5-9. Within a selenoprotein, means without a common letter differ ($P < 0.05$). Selenoproteins are not shown if they do not have any treatment effect. Gpx2, glutathione peroxidase 2; Gpx4, glutathione peroxidase 4; Txnrd 3, thioredoxin reductase 3; Sel15, the 15 kDa selenoprotein; Selt, selenoprotein T; Selk, selenoprotein K; Sels, selenoprotein S; Sephs2, selenophosphate synthetase 2; Selv, selenoprotein V.

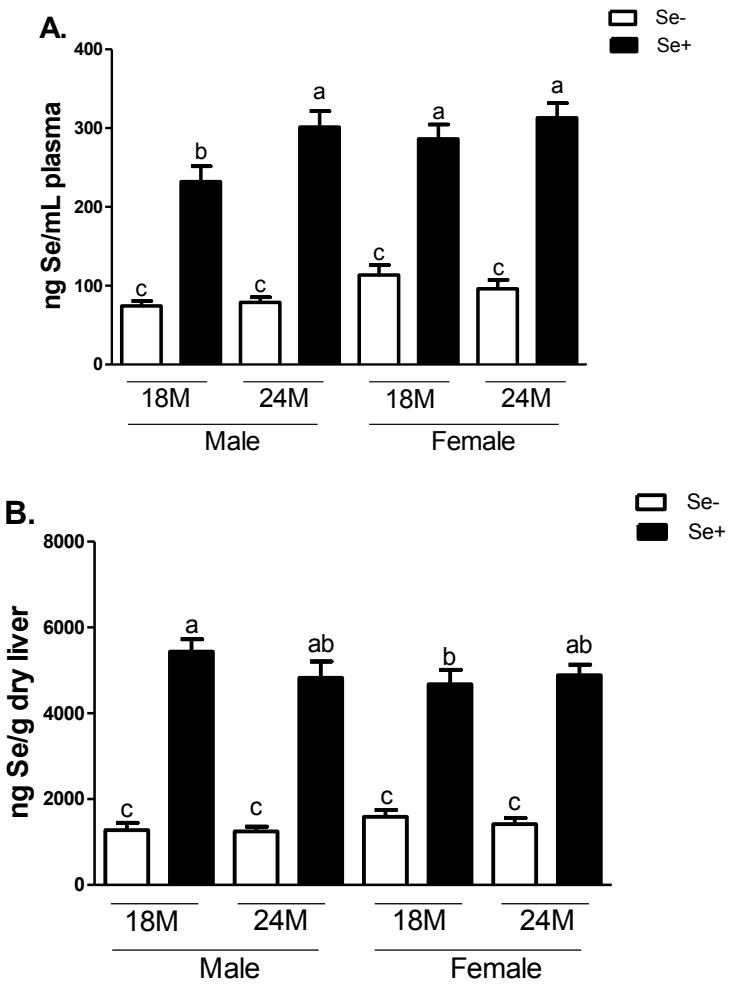

Figure 2.6 Effects of age and dietary Se deficiency on plasma (A) and liver (B) Se concentrations

Se concentration in G3 *Terc*$^{-/-}$ mice was determined by a graphite furnace atomic absorption spectrophotometry with Zeeman-effect background correction. Data are means ± SEM, n = 5-9. Within the same sex, means without a common letter differ, $P < 0.05$.

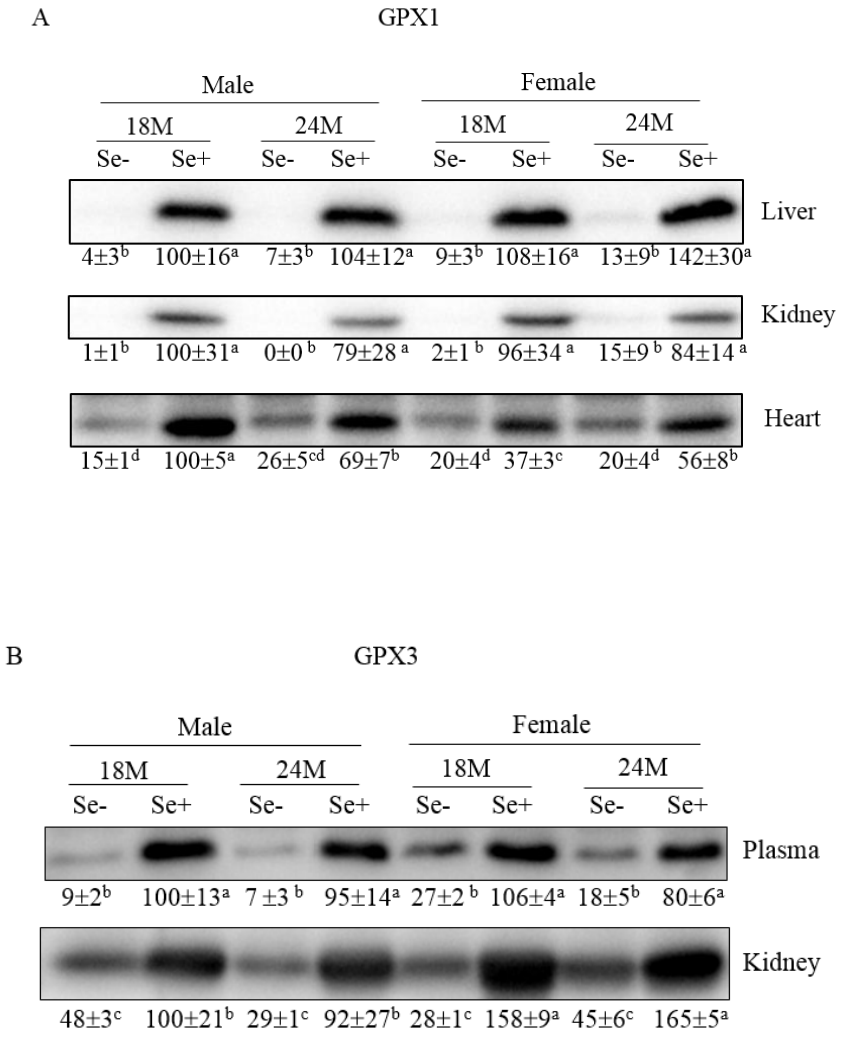

Figure 2.7 Effect of age and dietary Se deficiency on Gpx1 (A) and Gpx3 (B) protein expressions

Effect of age and dietary Se deficiency on GPX1 (A) and GPX3 (B) levels in various tissues of male and female G3 *Terc*$^{-/-}$ mice at 18 and 24 months of age determined by Western analyses. Values below the protein band were normalized by their respective actin bands and are means ± SEM, n = 3. Means without a common letter differ, $P < 0.05$. GPX1, glutathione peroxidase 1; GPX3, glutathione peroxidase 3.

CHAPTER III

EFFECTS OF OVEREXPRESSED SELENOPROTEIN H ON OTHER SELENOPROTEINS AND RESISTANCE TO OXIDATIVE STRESS IN 293T CELLS

3.1 Abstract

SELH mRNA shows great sensitivity to Se deficiency in various tissues and declines during aging in certain tissues. Deficiency of *SELH* in primary cells can accelerate the onset of replicative senescence. To study the interactions between *SELH* and other selenoproteins and the protective effects of this nuclear proteins on oxidative stress, a plasmid expressing wild-type SELH was constructed. Due to the possible deficiency of Se in the culture medium, various concentrations Na_2SeO_3 were added into the complete culture medium. Supplemental Na_2SeO_3 could increase the protein expression of SELH, while *SELH* transcript coded by plasmid shows insensitivity to Se concentration. However, without supplemental Na_2SeO_3, SELH reduced the protein level of two other selenoproteins, GPX1 and TrxR1, which are important antioxidant enzymes. But their mRNA expressions were resistant to SELH overexpression. In the presence of additional Na_2SeO_3, SELH showed the most significant protection against oxidative stress-induced loss of cellular viability and stress-induced senescence. SELH selectively increases the transcription of some heat shock proteins (HSP) regardless of additional Na_2SeO_3. Prediction based on the protein sequence suggests a high probability of the

existence of phosphorylation sites on SELH. Nevertheless, no phosphorylation site on SELH could be detected experimentally, with or without H_2O_2 challenge. LC-MS-MS analysis of proteins associated with SELH indicated the binding of SELH to ribosomal proteins.

3.2 Introduction

As an essential trace element, Se exerts its biological functions mainly through selenoproteins (22). Selenoproteins are a group of proteins that contain Sec. Sec is an analog of cysteine, with sulfur being replaced by Se. Sec is usually located at the active sites of the selenoproteins (2). The codon for Sec is UGA, which is usually utilized as a stop codon. In the presence of the SECIS element at the 3'-UTR of selenoprotein mRNAs, and other trans-acting factors, this UGA codon would be decoded as Sec and the translation will be continued until reaching their actual stop codon (2). However, when Se is limiting, Sec for the selenoprotein synthesis becomes insufficient and then the premature termination usually occurs. Thus, full-length selenoproteins cannot be generated. Humans have 25 selenoproteins, while rodents have 24 (19). The sensitivity of their RNA products and protein products to the deprivation of Se varies, resulting in selenotranscriptome and selenoproteome hierarchies under Se deficiency condition (123).

Almost all selenoproteins are oxidoreductases, including SELH (2). SELH was first identified from bioinformatics studies (19), and later found to be a small nucleolar protein with a molecular mass around 14 kDa (37). SELH is composed of 122 amino acids, and the Sec residue is located at the 44^{th} amino acid (19). The existence of *SELH* widely crosses species. It has been found in mammals, insects, and plants, but not in prokaryotes (22, 36). Compared to most other selenoproteins, *SELH* mRNA is very

sensitive to deficient Se intake (28, 39). How SELH responds to depletion of Se is not fully understood. SELH contains the conserved CXXU (C, Cys; X, any amino acid; U, Sec) motif, which may be a reversible thiolselenol/selenylsulfide redox group (37). Therefore, it belongs to the thioredoxin-like Family, along with SELT, SELV and SELW (42). The functions of SELH has been studied using knockdown and overexpression cell models (37, 40, 43). The known functions of SELH from these cell studies include DNA-binding, redox regulation, and suppressing cellular senescence (37, 40, 43). A study on *Drosophila melanogaster* showed that inhibition of *SELH* reduces the embryotic viability (35). Another recent study using zebrafish showed that mutant SELH can cause defected organ development and interact with p53 deficiency to accelerate tumor development (44).

In this present study, FLAG-tagged wild type SELH was transiently overexpressed in HEK 293T cells and evaluated how this transgene product interacts with other proteins and affects cellular responses to oxidative stress. Due to the possible deficiency of Se in the culture medium, various concentrations of Na_2SeO_3 was added into the complete culture medium. Through the studies described below, a deeper understanding of this nuclear selenoprotein is provided.

3.3 Materials and methods

3.3.1 Construction of expression plasmid

An expression plasmid encoding SELH was constructed using pFLAG-CMV™-4 Expression Vector (Sigma-Aldrich, St. Louis, MO). Human neuron cell cDNA (ScienCell Research Laboratories, Carlsbad, CA) was used as PCR cloning template. The cloning primers for SELH were consisted of the leader sequence, a restriction site, and

hybridization sequence. The nucleotide sequence for the forward cloning primer is 5'-CGGAATTCAATGGCTCCCCGCGGGAGG-3' and for the reverse cloning primer is 5'-GCTCTAGATTCAGGATCATGACTGAAGTTTGG-3'. The PCR product was purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), followed by insertion into the *EcoRI* and *XbaI* sites of pFLAG-CMV™-4 expression vector to generate the SELH expression plasmid (FLAG-SELH). The constructed plasmid was transformed into MAX Efficiency DH5a Competent Cells (Thermo Fisher Scientific) and cultured in LB medium containing 100 μg/ml ampicillin. The plasmid was then extracted using PureLink™ HiPure Plasmid Filter Purification Kits (Thermo Fisher Scientific). Sequencing was conducted by Eurofins MWG Operon (Louisville, KY) to confirm the insertion.

3.3.2 Cell Culture and Transfection

Human Embryonic Kidney 293T cells were purchased from ATCC (Manassas, VA) and cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. Cells were seeded one day before transfection in DMEM with 10% FBS. The cells were transfected with plasmids using X-tremeGENE 9 DNA Transfection Reagent (Roche, Indianapolis, IN), diluted in Opti-MEM (Thermo Fisher Scientific). Na_2SeO_3 at the indicated concentrations was added 6 hours after transfection. Na_2SeO_3 was chosen as the supplement because previously it has been found to be a more effective source of Se for selenoprotein synthesis for cells in culture than selenate or Se-Met (124).

3.3.3 Protein expression analysis by Western blot

The cells were sonicated in 2× Laemmli sample buffer (100mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 5% 2-mercaptoeethanol) twice for 2 sec each time then boiled at 95°C for 5 min. After cooled down to room temperature, cell lysis was resolved on an SDS-PAGE gel and transferred to a PVDF membrane using Tran-Blot® SD semi-dry transfer cell (Bio-Rad, Hercules, CA). Membranes were blocked in 5% non-fat milk solution for 1 hour at room temperature and incubated with primary antibodies at 4°C overnight. Primary antibodies included anti-β-ACTIN (sc-8432, Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG (F1804, Sigma-Aldrich), anti-GPX1 (GTX116040, GeneTex, San Antonio, TX), anti-TrxR1 (#15140, Cell Signaling Technology, Danvers, MA), and anti-TrxR3 (#12029, Cell Signaling Technology, Danvers, MA). The membranes were washed three times for 5 min each in TBS with 0.05% Tween-20 and then incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. The membranes were again washed three times for 5 min each and images were taken using ChemiDoc Imaging System (Bio-Rad). The quantification of the bands was conducted using Image Lab (Bio-Rad).

3.3.4 mRNA expression analysis by real-time PCR

Total RNA was extracted from cells using TRIzol® RNA isolation reagent (Thermo Fisher Scientific). Reverse transcription was performed using high capacity cDNA synthesis kit (Thermo Fisher Scientific). Real-time PCR analysis was conducted using iQ™ SYBR® Green Supermix (Bio-Rad) and 7500 Fast Real-Time PCR System (Applied Biotechnology, Foster City, CA). Primers used for real-time PCR are listed in Table 3.1.

3.3.5 Co-Immunoprecipitation

Cells were washed with PBS then lysed in a lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% TRITONÒ X-100) containing protease and phosphorylase inhibitors (Thermo Fisher Scientific). The cell lysate was centrifuged at 4°C for 10 minutes at 12,000 × *g*. Anti-mouse Protein A agarose beads were added into supernatant and incubated at 4°C for 1 hour. The beads were removed by centrifugation at 5,000 × *g* for 2 min at 4°C. Anti-FLAG M2 affinity gel was added and incubated at 4°C overnight. The resin was washed with a buffer (50m M Tris-HCl, pH 7.4, with 1 M NaCl) three times at room temperature for 5 min each. The bound proteins were eluted with 3×FLAG peptide.

3.3.6 Clonogenic assay

Cells were transfected and treated with Na_2SeO_3, and then trypsinized and counted with a hemocytometer. Three hundred cells were seeded into a 6-cm dish. H_2O_2 or paraquat at indicated concentrations was added to the cells 12 hours later and left for 24 hours before changed to fresh complete medium. Six days later, colonies were counted and one colony was defined as one containing more than 50 cells (125).

3.3.7 Phosphorylation detection

Proteins were enriched and purified using anti-FLAG immunoprecipitates. The eluted proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. Phosphorylation of FLAG-SELH was detected using primary antibodies including anti-phosphoserine, anti-phosphotyrosine, and anti-phosphothreonine (ab9332, ab10321, and ab9337, Abcam, Cambridge, MA).

3.3.8 Senescence-associated beta-galactosidase (SA-β-gal) detection

After transfection and Na_2SeO_3 treatment, cells were treated with 20 μM H_2O_2 for 4 hours and then changed into fresh medium. Cells were grown in fresh medium for another 6 days and then stained using a Senescence Detection Kit (Biovision, Milpitas, CA). After incubated at 37 °C overnight, the pictures were taken at 200× magnification using xxx inverted microscope (Wilovert A, Hund, Wetzlar, Germany).

3.3.9 Protein identification by LC-MS-MS

Proteins were enriched and purified using anti-FLAG immunoprecipitates. The LC-MS-MS analysis was performed as previously described (126). Spectral data were collected using an Orbitrap LTQ Velos mass spectrometer (Thermo Fisher Scientific) linked with an UltiMate 3000 nano flow HPLC system (Thermo Fisher Scientific). After tryptic digestion, proteins were loaded on reversed phase fused silica Acclaim PepMap C18 column measuring 75μm x 150mm (Thermo Fisher Scientific). The constant flow rate of 0.3 $\mu L/min^{-1}$ was used to separate and elute peptides via 60-minute long linear gradient of acetonitrile (in 0.1% formic acid): 2%-55% for 35 min, 95% for 10 min, 2% for 15 min. Peptides were detected by linear trap mass detector, in data-dependent acquisition mode with dynamic exclusion being applied. Eight scan events were employed: one MS scan (m/z range: 300–2000) followed by 7 MS/MS scans for the seven most intense ions detected in MS scan. Selected parameters were set as follows: normalized collision energy: 35%, automatic gain control (AGC) "on" with MSn Target 4×10^4, isolation width (m/z): 1.5, capillary temperature 170^{o}C, and spray voltage 1.97 kV.

The .raw files were searched using the SEQUEST algorithm of the Proteome Discoverer 1.1 software (Thermo Fisher Scientific) with given parameters: lowest and highest charge: +1 and +3, respectively; minimum and maximum precursor mass: 300 and 6,000 Da, respectively; minimum S/N ratio: 3; enzyme: trypsin; maximum missed cleavages: 2, FDR= 0.01; dynamic modifications: cysteine carbamidomethylation (+57.021), methionine oxidation (+15.995), methionine dioxidation (+31.990).

The spectral data were matched against the target *Homo sapiens* protein database obtained from the NCBI (www.ncbi.nlm.nih.gov). The reversed copy (created automatically by software) served as a decoy database.

3.3.10 Statistical analyses

Data were represented as means ± SEMs. Student *t* test was conducted using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) to compare the difference between two groups. *P* values less than 0.05 were considered significant.

3.4 Results

3.4.1 Expression of SELH in 293T cells increased by sodium selenite

An expression plasmid encoding *SELH* with N-terminal fused FLAG tag (pCMV-FLAG-SELH) was constructed to investigate the characteristics of SELH. DNA sequencing result of the constructed plasmid confirmed successful insertion of *SELH* from its start codon to its SECIS element located at 3'-UTR. Western blot analysis of 293T cells transfected with pCMV-FLAG-SELH showed one band with expected molecular mass when probed using anti-FLAG (Figure 3.1A). Cells transfected with empty vector (EV) were used as a control. The major resource of Se in culture medium

comes from fetal bovine serum. However, the Se in the culture medium may not be enough for full selenoprotein expression (127). Supplementation of Na_2SeO_3 into the cell medium after transfection enhanced the protein expression of FLAG-SELH in a dose-dependent manner, and quantification results showed that its plateau was reached at 100 nM (Figure 3.1B). However, at the transcription level, an increase by Na_2SeO_3 was only seen on endogenous *SELH* mRNA but not on plasmid encoded *SELH* mRNA (Figure. 3.1C).

p53 is suggested to play a central role in tumorigenesis and senescence in response to various types of cell stress (128). Furthermore, p53 has been recently found to be activated by *Selh* deficiency in zebrafish (44). Nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a transcription factor that facilitates the expression of genes containing antioxidant response element in their promoters. NRF2 activation and nuclear translocation are stimulated by NRF2 phosphorylation (129). Knockdown of *Selh* is known to stimulate phospho-NRF2 (40). However, neither SELH nor supplemental Na_2SeO_3 has any impact on p53 protein expression. Similarly, overexpression of SELH or additional Se in our study did not change levels of phospho-NRF2.

3.4.2 Effects of SELH overexpression on the expression of other selenoproteins

Overexpression of selenoprotein has been widely used to study their functions. Due to the insufficient Se in the culture medium, supplemented Se may be necessary to optimize the expression of overexpressed selenoproteins. What is more, the interactions between SELH and other selenoproteins also need to be elucidated. Here, these interactions at both mRNA and protein levels have been studied.

GPX1 and TrxR1 are well studied selenoproteins protecting cells against oxidative stress (2). When SELH was overexpressed, protein levels of both GPX1 and TrxR1 were reduced by approximately 50% and 30%, respectively. Adding Na_2SeO_3 increased protein levels of both GPX1 and TrxR1 (Figure 3.2). Both selenoproteins were saturated at10 nM Na_2SeO_3 in EV-transfected cells, but not saturated until 50 nM in FLAG-SELH overexpressed cells. Meanwhile, levels of TrxR3 protein did not change by either FLAG-SELH transfection or supplemental Se to the medium. TrxR3 protein expression was maximized by Se in the serum while overexpression of FLAG-SELH did not reduce its abundance. Thus, TrxR1 is more sensitive to limiting Se than TrxR3 at protein levels. In contrast, levels of *TXNRD1* and *TXNRD2* mRNA respond differently. In the absence of supplemental Se, FLAG-SELH did not affect mRNA level of any of the selenoproteins tested (Figure 3.3), which indicated that the decreased levels of GPX1 and TrxR1 protein were not caused by reduced mRNA levels. Instead, it is very likely caused by competition of Se with FLAG-SELH at the translation level. Adding Na_2SeO_3 increased mRNA abundances of *GPX1*, *SELW*, and endogenous *SELH* (Figure 3.3), which have been proven to be very sensitive to Se deficiency (28, 29). Unlike its protein product, *TXNRD1* mRNA was not sensitive to supplemental Se to cell culture medium. These results suggested that the changes at selenoprotein mRNA and protein levels are not always paralleled. *TXNRD2*/TrxR3 were insensitive to FLAG-SELH expression and supplemental Se at mRNA or protein levels.

3.4.3 Effects of SELH on genes with HSE

Through chromatin immunoprecipitation assay, Panee *et al.* showed that SELH bound heat shock element (HSE) and stress response element (43) in the promoter

regions, but how such protein-DNA interactions affected the subsequent expression was not clear. Levels of four heat shock proteins (*HSP*s), *HSP27*, *HSP70-1A*, *HSP70-1B,* and *HSP90AA1* mRNAs were tested, whose gene promoters containing HSE, under the condition of SELH overexpression and Se supplementation. While only *HSP70-1B* mRNA level was enhanced by Na_2SeO_3 supplementation in EV-transfected cells, *HSP27*, *HSP70-1A*, and *HSP70-1B*, but not *HSP90AA1 mRNA* levels were increased by FLAG-SELH overexpression (Figure 3.4). *HSP27* had the greatest increase by SELH among all these four genes tested. The increased level of *HSP70-1B* mRNA caused by 100 nM Na_2SeO_3 was similar with that by FLAG-SELH. The mRNA of the chosen *HSPs* were collected at 37°C, no heat stress or other external stimuli were applied. Overall, the increased mRNA level of *HSP* induced by FLAG-SELH is gene specific. Although these four HSPs contained HSE in their promoters, the numbers of HSE, their locations, specific sequences, and the associated transcription factors are different (130-133).

3.4.4 Protection of SELH against H_2O_2 and a pro-oxidant, paraquat

Our lab has previously shown that knockdown of SELH increases cellular sensitivity to H_2O_2 and paraquat, but not other DNA-damaging agents (40). Here whether overexpressed SELH in 293T cells protected against H_2O_2 and paraquat has been tested using clonogenic assay. The results showed that in the absence of supplemental Se, overexpressed SELH did not provide resistance to H_2O_2 or paraquat treatment (Figure 3.5). When 100 nM Na_2SeO_3 was added, these cells showed increased resistance to H_2O_2 regardless of EV or FLAG-SELH trasnfection. However, in the presence of 100 nM Na_2SeO_3, compared with EV, cells overexpressed with SELH showed significantly increased survival in the cellular response to H_2O_2 treatment. They were completely

resistant to 20 μM H_2O_2. In contrast, the protection against 20μM paraquat was only seen under overexpressed SELH and 100 nM Na_2SeO_3 supplement combined condition.

3.4.5 Effect of SELH on stress-induced senescence

SA-β-gal has been widely used as a marker of cellular senescence. In this study, HeLa cells were used to determine the effect of FLAG-SELH overexpression on H_2O_2-induced senescence. Both in the absence and presence of 100nM Na_2SeO_3, FLAG-SELH decreased the numbers of H_2O_2-induced SA-β-gal positive cells. In addition, when Hela cells were supplemented with Se alone, the SA-β-gal positive cell level was also reduced upon H_2O_2 treatment (Figure 3.6).

3.4.6 Phosphorylation detection using antibodies

Phosphorylation sites on human SELH were predicted using NetPhos 3.1 Server (Technical University of Denmark). Multiple sites on SELH were predicted as targets of phosphorylation (Figure 3.7). Since post-translational modifications are more likely to occur with external stimuli instead of normal growth condition and SELH protects against oxidative stress, acute H_2O_2 challenge was applied to determine if SELH phosphorylation could be stimulated. Immunoprecipitation assays using anti-FLAG resin and elution with 3×FALG peptide were performed to enrich SELH protein. Western blot analyses employing antibodies against phosphoserine, phosphothreonine, and phosphotyrosine did not result in detectable bands under normal growth condition or treated with acute H_2O_2 in 293T cells. Thus, SELH appears not be phosphorylated.

3.4.7 Effects of a CRL2 inhibitor on SELH protein expression

Proteasome-mediated degradation is a major pathway to eliminate the misfolded proteins (134). Two proteasome inhibitors, MG132 and lactacystin (Lac), were dissolved in DMSO and used to suppress the proteasome degradation pathway and test whether suppression of proteasome pathway would affect the ectopic expression of SELH protein. The Western blot results showed that inhibition of proteasome pathway could not inhibit the degradation of SELH (Figure 3.8), indicating that proteasome may not be responsible for the degradation of SELH.

Short form of prematurely terminated FLAG-SELH, whose calculated molecular mass was around 5 kDa, was undetectable by Western blot in our study. Thus, weather the prematurely terminated form of SELH under the regulation of proteasome degradation pathway could not be tested.

The cullin-RING ubiquitin ligases (CRLs) are multi-subunit E3 ligases, which ubiquitinate protein substrates for degradation (135). Among numerous CRLs, CRL2 has been identified to selectively participate in the degradation of certain prematurely terminated selenoproteins (136). Cullin is a major subunit of CRL, and posttranslational neddylation of cullin is required for activation of CRLs. MLN4924 is a known inhibitor of CRL through cullin neddylation (135). To block CRL function, transfected 293T cells were treated with 1 μM MLN4924 (Fisher Thermo Scientific) for 6 hrs. Prematurely terminated form of SELH could not be detected in the presence of MLN4924. Results from Western blot analyses showed that inhibition of CRL2 activity by MLN4924 treatment did not affect the expression of full-length SELH.

3.4.8 Identification of SELH associated protein by immunoaffinity enrichment coupled with LC-MS-MS

To identify SELH-associated proteins, FLAG-SELH was overexpressed in 293T cells in the presence of 100 nM Na_2SeO_3 supplementation to the medium. Anti-FLAG M2 agarose affinity gel was used to pull down FLAG-SELH and its associated proteins by co-immunoprecipitation. A silver staining gel showing proteins eluted from the affinity resin was displayed in Figure 3.9. Single gel band as labeled was cut and prepared for trypsin digestion. Trypsin-digested proteins were then identified by tandem mass spectrometry sequencing. Surprisingly, unlike only one band was identified by anti-FLAG using Western blot, two protein bands were identified as SELH by tandem mass spectrometry sequencing, a short form with approximate 14 kDa and a long form with approximate 16 kDa. Based on the molecular mass, it is unlikely that the short form was the prematurely terminated protein. The nature of these two SELH isoforms warrant further investigation.

Among those proteins identified to associate with FLAG-SELH were ribosomal proteins (Figure 3.9), including 60S ribosomal proteins L8 and L3 and 60S acidic ribosomal protein P0. Their main subcellular locations are nucleolus (37). The finding that ribosomal proteins were associated with FLAG-SELH is consistent with the previous study using CxxC or CxxS mutant SELH (37). In contrast to this study (37), no thiol oxidoreductases has been identified in this study. Another interesting finding is that galactoside 2-α-L-fucosyltransferase 2 was associated with SELH. This suggests that SELH may be capable of being glycosylated. Another associated protein, zinc fingers and homeoboxes protein 3, is a transcription repressor highly expressed in the kidney (137). The differences between the group of proteins identified in this study and the group of

proteins identified in the previous study (37), may come from the different forms of SELH used (mutant vs. wild type), the different Se concentrations in the media, and the different cell lines used for SELH expression.

3.5 Discussion

This study revealed an interesting interaction between SELH and other selenoproteins and suggested that Se availability in cell culture medium is a limiting factor for optimized selenoprotein expression when over-expressed. Possible regulations and modifications of SELH were also tested.

In our study, additional Se significantly increased the protein expression of SELH. Unlike endogenous *SELH* mRNA, exogenous *SELH* mRNA abundance was not increased by the supplemental Se. Among all the identified selenoproteins, *SELH* mRNA is one of a few that are very sensitive to deficiency in Se availability (28, 39). One noticeable difference between exogenous and endogenous *SELH* mRNA is their 5'-UTRs and 3'-UTRs. The exogenous SELH encoded by the plasmid contains no 5'-UTR and its 3'-UTR stops at the SECIS element. It is known that both 5'-UTRs and 3'-UTRs can contain regulatory elements that regulate mRNA transcription, mRNA stability, and translation (138). Characteristics of 5′-UTR that can affect transcription include length, conserved sequences that function as binding sites for regulatory proteins, and the presence of secondary structure. Similarly, 3′-UTRs contain numerous binding sites for regulatory factors; these factors are usually proteins, but in a few cases *trans*-acting RNAs have been described (138). Also, both 5'-UTR and 3'-UTR can be targets of microRNA binding. MicroRNAs are a family of small non-coding RNAs known to post-transcriptionally regulate target mRNAs. It can suppress mRNA translations but also can

induce mRNA cleavage (139). Since *FLAG-SELH* does not contain 5'-UTR and its 3'-UTR ends at SECIS element, its transcription and degradation regulations are very likely to be different with endogenous *SELH*. Another possibility involves one mRNA degradation pathway, NMD, which has been proven to selectively participate in the degradation of some selenoprotein mRNAs under Se deficiency condition (30). A currently accepted hypothesis proposes that only transcripts characterized with a premature termination codon of at least 50–55 nucleotides upstream of an exon junction are vulnerable to NMD (140). Based on this, *SELH* is predicted to be a target of NMD (30). However, so far no experimental results show whether NMD regulates *SELH* mRNA or not. Although the occurrence of NMD does not require 5'-UTR or 3'-UTR, exon-junction complex is needed to trigger the targeted mRNA decay (141). Since no splicing would happen on this exogenous *SELH* mRNA, exon-junction complex cannot mark it for degradation. If NMD is responsible for the sensitivity of endogenous *SELH* mRNA to Se deficiency, the exogenous *SELH* cannot be regulated by this degradation pathway.

Our findings showed that in the absence of additional Se, overexpression of SELH causes decreased protein expression of two major selenoproteins GPX1 and TrxR1, while supplementing with Se could rescue this decreased protein abundance. One possible explanation might be overexpressed SELH depletes the available Se in the culture medium, and additional Se makes it enough for the translation of all three selenoproteins. *GPX1* mRNA is supposed to be very sensitive to the Se status (28, 30, 39). In our study, the supplemental Se enhances both mRNA and protein expression of *GPX1*. However, the changes of GPX1 protein and mRNA levels may not always be

paralleled (142, 143), which is consistent with the overexpressed SELH study. In contrast to the responses at protein levels, mRNA expression of *GPX1* and *TXNRD1* were resistant to overexpressed SELH. Considering the Se and time needed for the expression of plasmid-coded SELH, the change of medium Se caused by overexpressed SELH is likely to be more subtle and happen at a much slower rate compared with adding Na_2SeO_3 into the medium. This possibility is supported by results of GPX1 protein changes. Adding 10 nM Na_2SeO_3 increased GPX1 protein expression by around 170%, while overexpressed SELH decreased GPX1 mRNA expression by approximately 50%. The more subtle level change of GPX1 protein by overexpressed SELH is consistant with a more subtle change of *GPX1* mRNA. Compared with *GPX1* mRNA, *TXNRD1* mRNA is less sensitive to Se status (28). Furthermore, it has been shown that *TXNRD1* mRNA is also less sensitive to Se than its protein (144). Thus it is not surprising to observe decreased TrxR1 protein expression in the absence of mRNA changes by overexpressed SELH.

Lately, a study on zebrafish showed that p53 is an important mediator for *Selh*, since dramatically elevated p53 levels was observed in *Selh* mutant larvae, and loss of *tp53* in *Selh* mutant fish could mitigate developmental defects caused by *Selh* mutant (44). In another study using primary fibroblasts, the phospho-NRF2 level was also found increased by knockdown of SELH (40). However, neither p53 nor phospho-NRF2 was changed by overexpressed SELH in our study. Lack of p53 response to overexpressed SELH is consistent with a previous study using murine neuron cells (46). Although the changes of protein expression of endogenous SELH by exogenous SELH could not be determined, it is unlikely to be as detrimental as those described in the aforementioned

two SELH deficiency studies. The exogenous SELH is also supposed to provide protective effects. No change of p53 or phospho-NRF2 protein expression by SELH suggests that unlike SELH deficiency, overexpressed SELH exerts its protective functions independent of p53 and phospho-NRF2 levels.

Knockdown of SELH resists cells to oxidative stress (40). However, expression of SELH alone could not offer maximized protection against oxidative reagents. When Se is sufficient, cells with overexpressed SELH showed increased resistance to cell death and senescence in response to oxidative stress. Adding Se alone cannot provide the same protective effects. The decreased expression of other selenoproteins such as GPX1 and TrxR1 caused by overexpressed SELH may explain the insignificant protective effects of SELH in the absence of additional Se. Both GPX1 and TrxR1 participate in the sensing and signaling of H_2O_2 and reduce it in a direct or indirect way (145). *Gpx1*$^{-/-}$ mice and primary fibroblasts showed increased sensitivity to H_2O_2 and paraquat (84, 146). TrxR1 functions as a suppressor of H_2O_2 and thus inhibition of TrxR1 could elevate intracellular H_2O_2 level in rheumatoid arthritis synovial cells (147). In addition, these two selenoproteins are known repressor of senescence. Fibroblasts derived from *Gpx1*$^{-/-}$ mice showed senescence-like features, such as reduced cellular proliferation rate and larger cellular surface area (84). Inhibition of TrxR1 could induce stress-induced senescence in murine fibroblasts (148). Considering the decreased protein expression of GPX1 and TrxR1 caused by overexpressed SELH, it is possible that when those selenoproteins with important redox regulation functions being decreased, the protective function of SELH might be compromised.

Our results show that SELH can selectively stimulate the mRNA expression of HSPs. HSPs are a group of proteins that can be induced by heat stress and may be important modifying factors in cellular responses to a variety of physiologically relevant conditions such as hyperthermia, oxidative stress, and aging (149). The HSP family encompasses multiple members sharing common features while having instinct cellular functions. For instance, *Hsp27* is involved in microfilament stabilization and anti-apoptosis; *Hsp70* participates in anti-apoptosis; and *Hsp90* regulates steroid hormone receptors and protein translocation (149). In the absence of heat stress, supplemental Se can induce mRNA expression of HSPs in chicken splenic lymphocytes and Wistar rat pup lenses (150, 151). Besides Se status, aging is another factor that affects HSPs. Aging in mammalian organisms involves degeneration of HSP expression with time (152). The heat shock response also declines over the lifetime, and the weakening is known to contribute to aging by permitting the emergence of protein aggregation diseases and reduction in cellular vitality (152). RNA expression of small HSPs is increased in *Drosophila* with a longer lifespan (153), and overexpression of *hsp70F* increases the lifespan of *C. elegans* (154). Recent findings show protective effects for HSP27 in Huntington's disease, a neurodegenerative disease (155). Considering the protective functions of HSPs, it is tempting to speculate that SELH may repress cellular senescence partially through up-regulation of HSPs. In addition, the expression promotion effect of SELH does not differ when additional Se is absent or present, indicating that this effect may not be compromised by the reduced level of other selenoproteins. Future studies could investigate whether SELH suppress senescence in an HSP-dependent or -independent manner.

Identification of SELH-associated proteins has been done using CXXS or CXXC mutants (37), instead of CXXU wild-type full-length SELH. Multiple nucleolar proteins and thiol oxidoreductases have been identified using these two mutants (37). In the current study, using wild-type SELH and LC-MS-MS sequencing, multiple proteins associated with SELH were found. The proteins identified to be associated with SELH supports its nucleolus subcellular location and indicates its possible glycosylation modification. Unexpectedly, two protein bands containing SELH peptides were found, according to sequencing results. One was around 2 KD larger than the other one. Based on molecular weight, the upper one was the one detected by anti-FLAG using Western blot. In contrast, the lower one could be detected only by immunoprecipitation but not Western blot. Alternative translation initiation is a common mechanism to generate protein isoforms. The FLAG-SELH open reading frame contains two ATG codons, one from expression vector and the other one from the inserted SELH coding region. The molecular mass difference between these two bands makes it tempting to speculate that the lower band was generated using alternative translation initiation, which used the ATG codon from SELH coding region instead of the ATG codon from the expression vector for translation initiation. Therefore, no FLAG tag would be contained in the synthesized protein. Given the molecular mass of the FLAG tag, which is calculated around 1.7 KD (MDYKDDDDKLAAANS), the lower band has the expected molecular mass of SELH with no N-terminal FLAG. However, since it can be detected using immunoprecipitation, it is very unlikely the lower band does not contain FLAG. Another possibility is that from the same mRNA, two forms of SELH are generated, similar with the diversity of MsrB1 protein forms (156). Nevertheless, the exact amino acid sequence of protein in the lower

band could not be determined; it is hard to make the conclusion whether it is a short form of SELH or not. The composition differences between these two isoforms are of great interest and need further investigation.

3.6 Tables and Figures

Table 3.1 Primers used for RT-PCR in SELH study

Gene	Forward	Reverse	Siz
β-ACTIN	GGACCTGACTGACTACCTCAT	CGTAGCACAGCTTCTCCTTAAT	107
SELH	GCTCTGGACTGGGATTAAGAA	GCTTCTACCCAAATCTCCCTAC	112
GPX1	AGGGAGGAACACCTGATCTTA	CTGACACCCGGCACTTTATTA	117
TXNRD1	GGACGATTCCGTCAAGAGATA	TTGGACCCAGTACGTGAAAG	99
TXNRD2	TTGACGAGCACACGGTTT	CCTCCAGTAGCAATGATGATGT	85
SELW	GCCGTCCGAGTCGTTTATT	GGGAACTCATCTTCTAACTTCTT	83
HSP27	AGGACGAGCATGGCTACAT	GGACAGGGAGGAGGAAACTT	92
HSP70-	CCACCATTGAGGAGGTAGATT	CAGGAAATTGAGAACTGACAAA	110
HSP70-	TGATGGTAATTAGCTGGCTTC	GGGAACGAAACACCCTTACA	100
HSP90AA	GGAGATAAACCCTGACCATTC	GACAGGAGCGCAGTTTCATA	117

SELH, selenoprotein H; GPX1, glutathione peroxide-1, TXNRD1, thioredoxin reductase 1; TXNRD2, thioredoxin reductase 2; SELW, selenoprotein W, HSP27, heat shock protein 27; HSP70-1A, heat shock 70 kDa protein 1A; HSP70-1B, heat shock 70 kDa protein 1B; HSP90AA1, heat shock protein 90 alpha family class A member 1

.

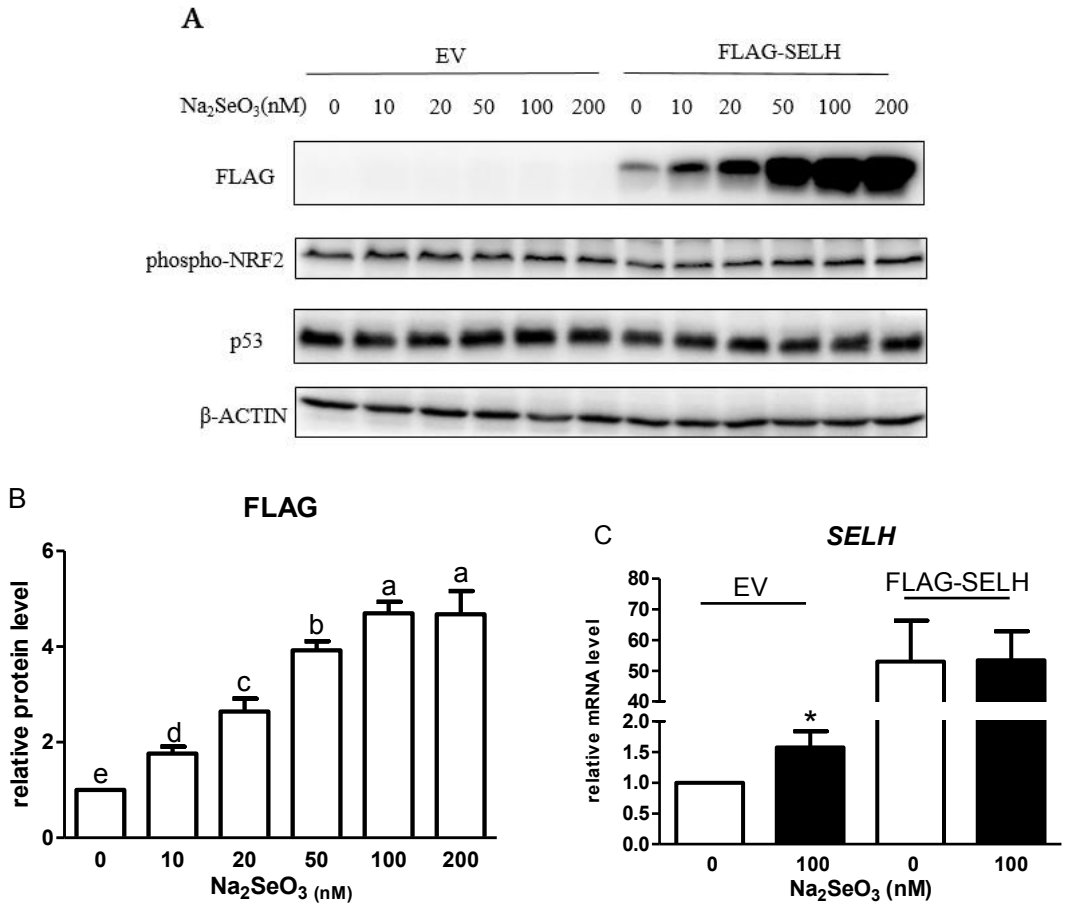

Figure 3.1 Na_2SeO_3 dose-dependent increases in the overexpression of FLAG-SELH

(A) Western Blot analysis of protein expression of FLAG-SELH, phospho-NRF2, P53, and β-ACTIN. Transfection and Na_2SeO_3 treatment were conducted as described in the materials and methods. (B) Quantification of the FLAG-SELH protein expressions in 293T cells when different concentrations of Na_2SeO_3 were added. The values were normalized by β-ACTIN. Results were represented as mean ± SEM. Labeled without a common letter means the difference is significant, $P < 0.05$. (C) RT-PCR analysis of mRNA expression of *SELH*. Results were from three independent experiments represented as mean ± SEM. * Different from same vector no Se control, $P < 0.05$. SELH, selenoprotein H; NRF2, Nuclear factor (erythroid-derived 2)-like 2.

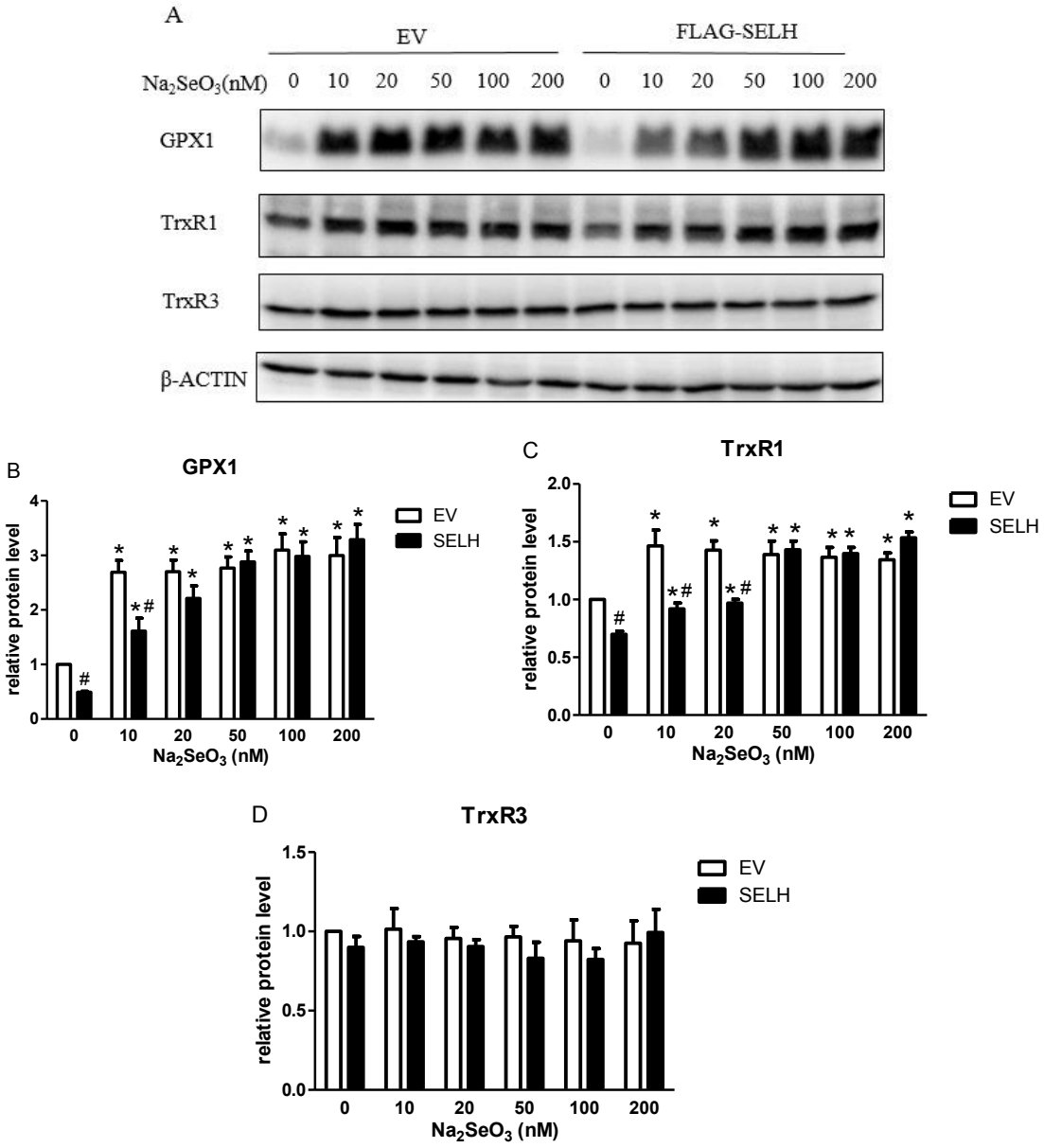

Figure 3.2 Effect of SELH overexpression and supplemental Se on protein levels of three other selenoproteins

Western Blot analysis of protein expression of (A) GPX1, TrxR1, and TrxR3 in 293T cells with indicated treatment. Transfection and Na_2SeO_3 treatment were conducted as described in the materials and methods. Quantification of (B) GPX1, (C) TrxR1, and (D) TrxR3 protein expressions. The values were normalized by β-ACTIN. Results were represented as mean ± SEM. * Different from same vector no Se control, $P < 0.05$. # Different from empty vector same Se concentration control, $P < 0.05$. GPX1, glutathione peroxide-1; TrxR1, thioredoxin reductase 1; TrxR3, thioredoxin reductase 2.

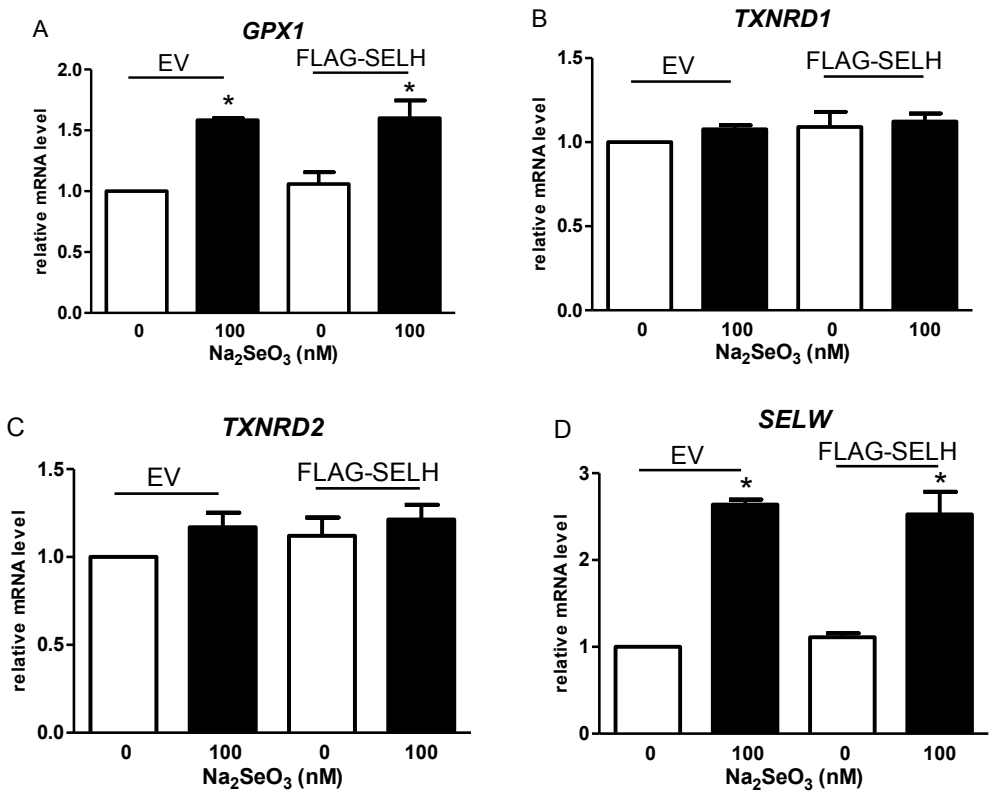

Figure 3.3 Effect of SELH overexpression and supplemental Se on mRNA expressions of other selenoproteins

RT-PCR analysis of (A) *GPX1*, (B) *TXNRD1*, (C) *TXNRD2*, and (D) *SELW* in 293T with indicated treatment. The values were normalized by β-ACTIN. Results were from three independent experiments and represented as mean ± SEM. * Different from same vector no Se control, $P < 0.05$. # Different from empty vector same Se concentration control, $P < 0.05$. GPX1, glutathione peroxide-1; TXNRD1, thioredoxin reductase 1; TXNRD2, thioredoxin reductase 2, SELW, selenoprotein W.

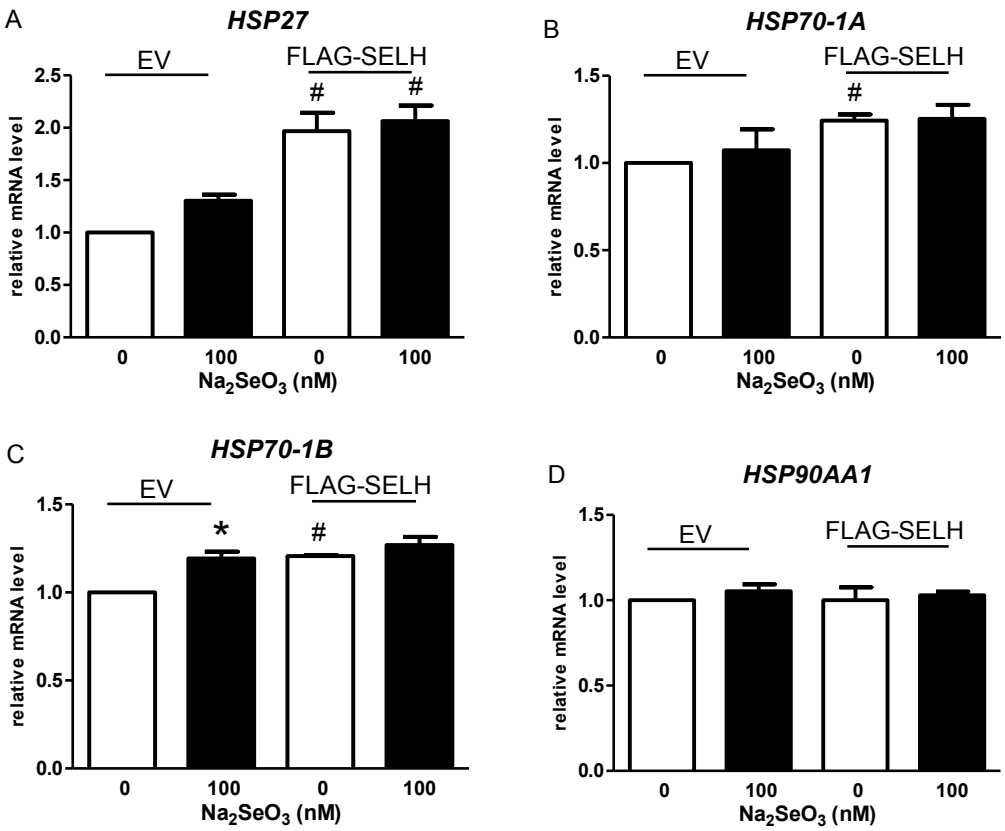

Figure 3.4 Effects of SELH overexpression and supplemental Se on mRNA levels of HSP

RT-PCR analysis of (A) *HSP27*, (B) *HSP70-1A*, (C) *HSP70-1B*, and (D) *HSP90AA1* in 293T with indicated treatment. The values were normalized by β-ACTIN. Results were represented as mean ± SEM. * Different from same vector no Se control, $P < 0.05$. # Different from empty vector same Se concentration control, $P < 0.05$.

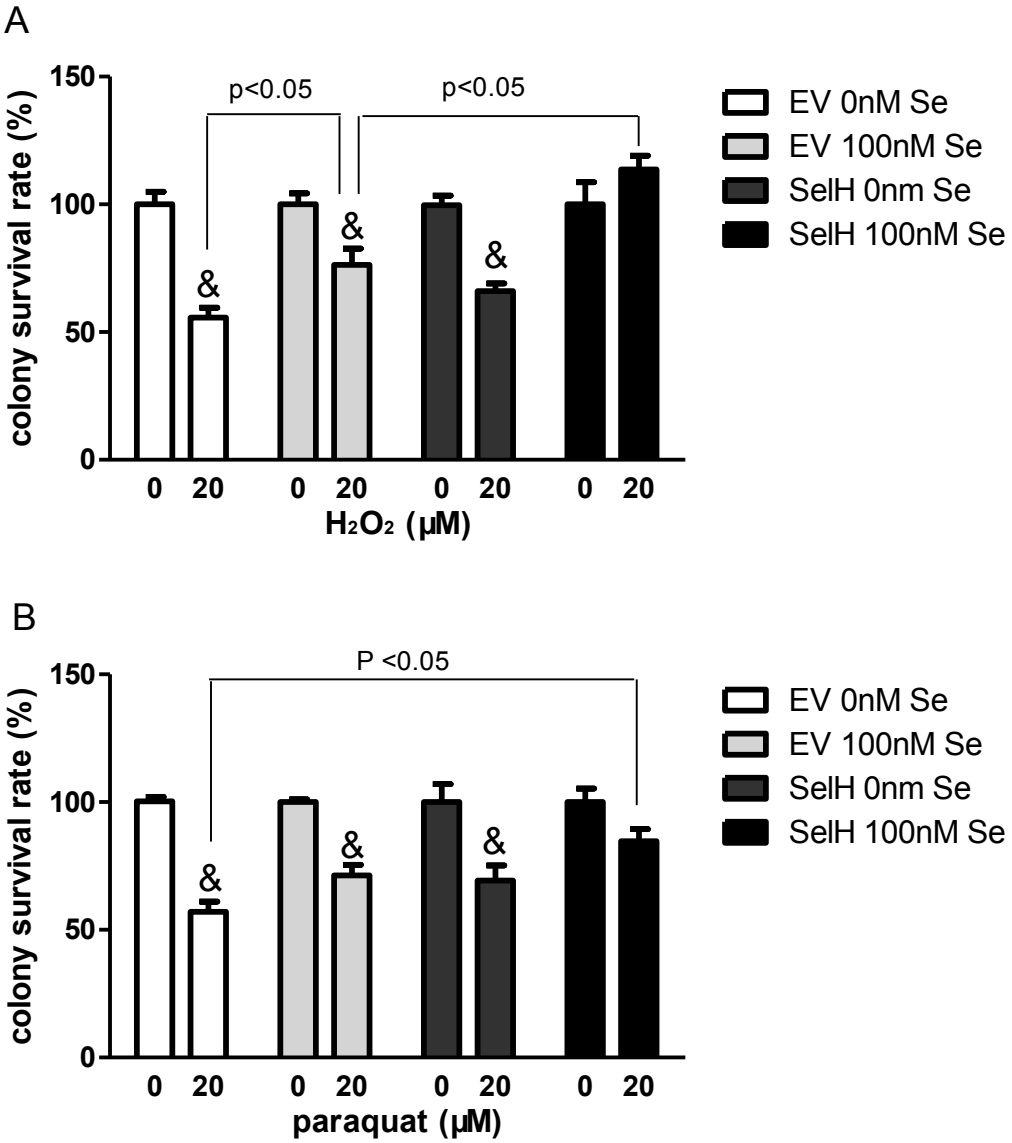

Figure 3.5 Effects of SELH overexpression and supplemental Se on cellular response to oxidative stress

Clonogenic assays were performed in 293T cells transfected with EV or FLAG-SELH following treatment with 20 μM H_2O_2 (*A*), or 20 μM paraquat (*B*). The survival rates were presented with mean ± SEM (n = 3). & Different from the same vector same Se concentration but no H_2O_2 control, $P < 0.05$.

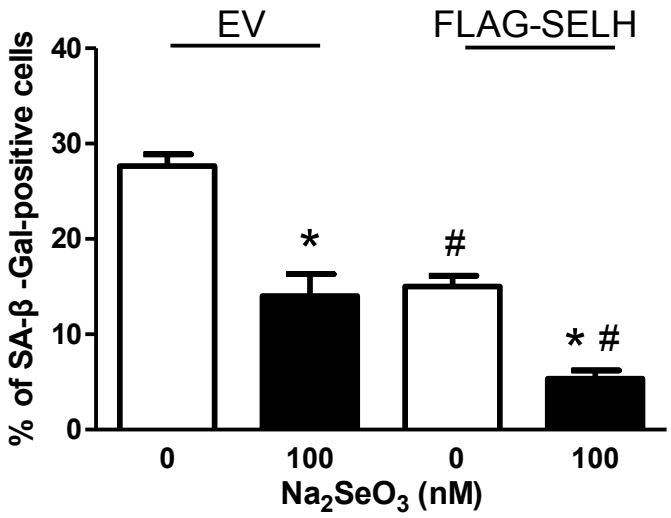

Figure 3.6 Effects of SELH overexpression and supplemental Se on the senescence response to H_2O_2

Transfected 293T cells were treated with 20 μM H_2O_2 for 4 hours then changed to fresh medium and grew for another 6 days. The SA-β-gal was stained overnight before the pictures were taken. (A) Representative images of treated cells. (B) Percentage of SA-β-gal-positive cells. * Different from same vector no Se control, $P < 0.05$. # Different from empty vector same Se concentration control, $P < 0.05$.

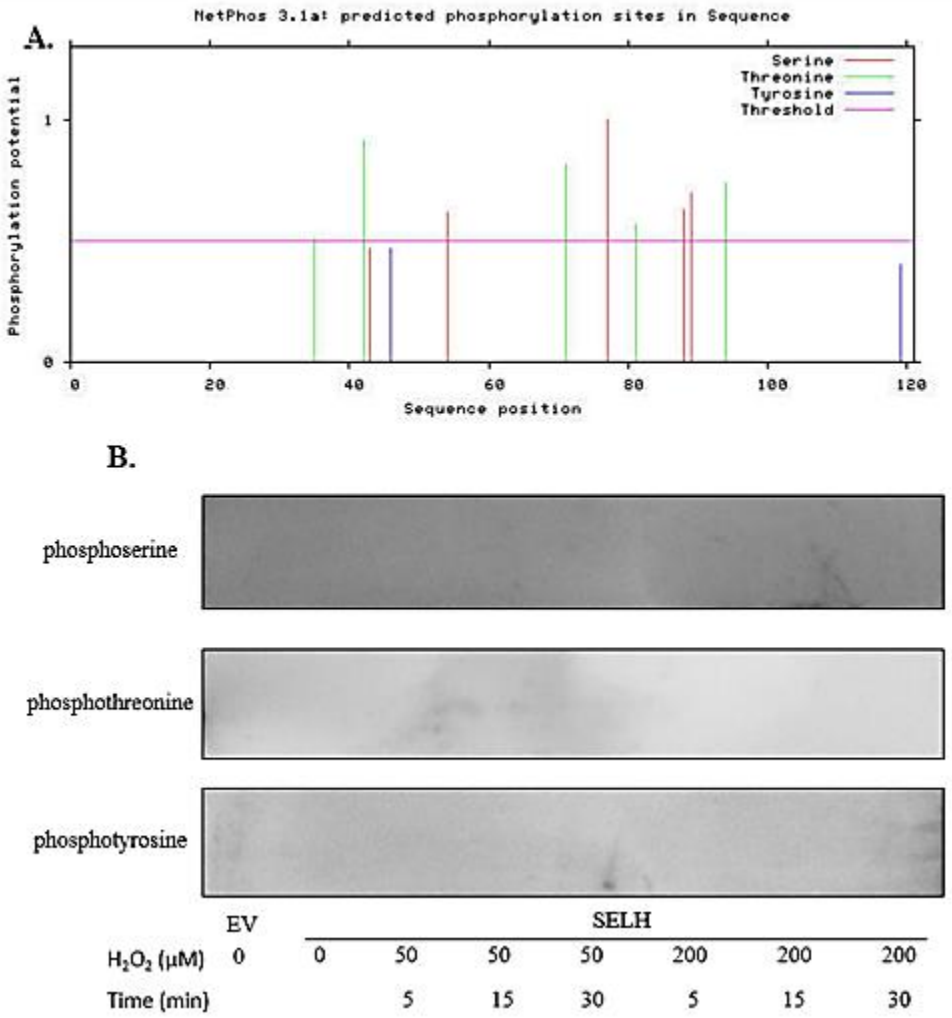

Figure 3.7 Phosphorylation prediction and detection of FLAG-SELH

(A) Phosphorylation possibility of Serine, Threonine, and Tyrosine residues predicted by the NetPhos 3.1. Blue, green and red lines correspond to the phosphorylation potential of Serine, Threonine, and Tyrosine residues, and the pink horizontal line indicates the threshold for the phosphorylation potential. (B) Detection of phosphorylation on SELH using immunoprecipitation and western blot. 293T cells were transfected with EV or FLAG-SELH, and the cells were harvested 36 h after transfection. The cell extracts were immunoprecipitated with anti-FLAG resins and probed with anti-phosphoserine, phosphothreonine, and phosphotyrosine antibodies.

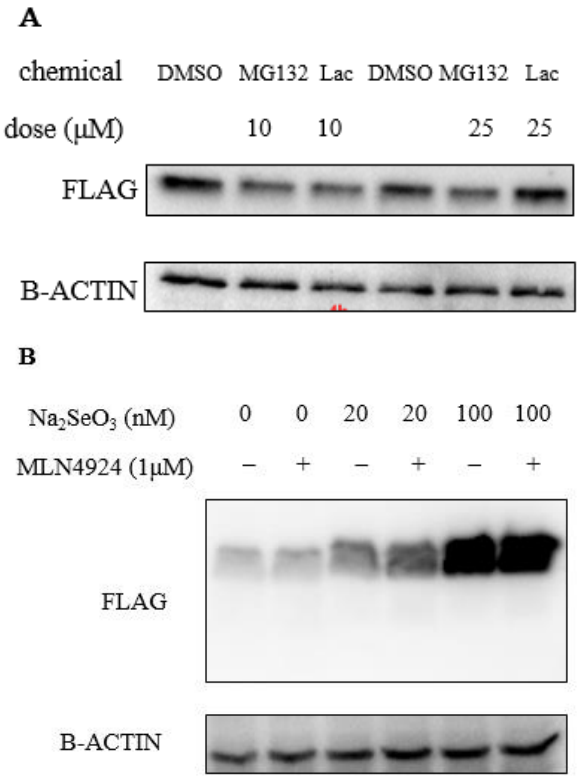

Figure 3.8 Effects of proteasome inhibitors and a CRL inhibitor on the expression of FLAG-SELH

FLAG-SELH transfected 293T cells were treated with (A) proteasomes inhibitors MG132 or Lac at indicated concentrations for 4 hours or (B) CRL inhibitor at 1μM for 6 hours.

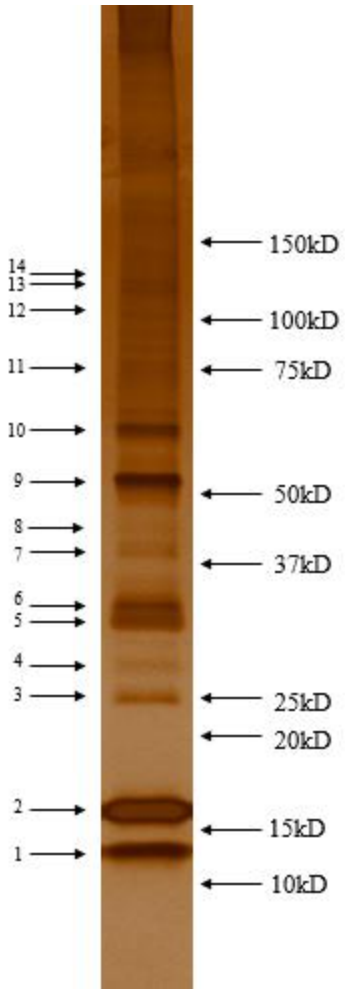

Figure 3.9 Identification of SELH-associated proteins in 293T cells

This silver stained SDS-PAGE gel shows proteins from FLAG-SELH expressing 293T cells eluted from FLAG resins. The following proteins were identified by MS/MS sequencing of protein bands extracted from the gel: 1 - short form of SELH, 2 - long form of SELH, 3 - pancreatic progenitor cell differentiation and proliferation factor isoform X1, 4 - 60S ribosomal protein L8, 5 – G4 protein, 6 - 60S acidic ribosomal protein P0, 7 - 60S ribosomal protein L3, 8 - galactoside 2-alpha-L-fucosyltransferase 2, 9 - katanin p60 ATPase-containing subunit A-like 1, 10 - protein FAM53C, 11 - zinc fingers and homeoboxes protein 3, 12- collagen alpha-1(XI) chain isoform X3, 13 - unconventional myosin-Ig (Specifically expressed in hematopoietic cells), 14 - nck-associated protein 5-like

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

The aims of this dissertation were to gain a better understanding of the selenoproteins playing in mediating lifespan and health-span in elderly subjects (65) and the protective effect of *Selh*, whose role in cellular senescence has been identified (40). The first project has been, due to our knowledge, the study using the longest Se deficiency diet treatment in animals. Se deficiency is relatively rare nowadays. Se-fortified food has been used to eliminate the deficiency in areas with low Se intake (1). However, the biological functions of the 25 selenoproteins in humans and 24 selenoproteins in rodents are not fully understood.

The common animal studies using Se-deficient and -adequate diets usually last from weeks to a few months (28, 88-90). The deprivation of Se on female rats could induce growth retardation in their offspring (157), and our previous study showed how life-long dietary Se deprivation impacts health-span and life-span of the mice (65). It provided us with new insights into the physiological functions of this trace element. The analysis of selenotranscriptome change during this aging process could also provide a better understanding of this aging phenomenon.

In Chapter II, the effects of lifelong dietary Se deficiency, sex, and aging on selenotranscriptomes in four tissues have been extensively studied. All 24 rodent selenoprotein mRNAs in the heart, kidney, liver, and testis were analyzed. The four

unique patterns discovered suggested that no single selenoprotein can explain all the age-related degeneration observed in those short telomere mice. Instead, multiple selenoproteins may work together, and their combined effects may confer the aging phenotypes observed. Besides directly affecting the selenoprotein expressions, dietary Se deprivation may exert its physiological effects through another way. Modulation of dietary Se concentrations for ten weeks is known to regulate gut microbiota in mice (158), which indicates that gut microbiota may also influence the aging process in our mice. It is of future interests to investigate the effects of long-term dietary Se deprivation, aging, and sex on gut microbiota composition, and determine how they mediate the aging process.

The results showed three selenoproteins, *Gpx1*, *Selh*, and *Selw*, whose mRNAs showed sensitivity to Se deficiency across all the tissues except testis. They have been previously found to be prone to decrease in response to Se deprivation in various tissues in young mice (28, 29). This study confirmed that they could be used as Se status marker even at different aging stages. Many selenoproteins respond to Se deprivation differently in different tissues and also have sexually dimorphic expressions (16, 76); however, *Selh*, *MsrB1*, and *Selo* have been identified in our study that their mRNA levels are down-regulated by aging in multiple tissues. However, due to the unparalleled changes of selenoprotein mRNA and protein, further studies is also needed to confirm the changes of many other selenoproteins at the translation level. Furthermore, the findings on aging effect were based on the comparison between mice at 18 and at 24 months old. Because the 18 months old mice could not be considered as young, it is very likely that 6 or 12 months old mice may have different expression pattern compared with 24 months.

Although future studies on comparisons between young mice and old mice may provide different insights, here our results suggest an age-dependent selenotranscriptomic changes in late life.

SELH was first discovered and identified as a selenoprotein through genome sequencing (19). When Se is deficient, mRNA expression of *Selh* in various tissues, such as liver, kidney, and colon (28, 29), decreases remarkably, making it one of a few ranked the lowest in the selenotranscriptome hierarchy. The abundance of *Selh* mRNA is high during embryonic stage in mice (37). *SELH* mRNA level decreases in senescent primary cells, compared with young counterparts (39). The study in Chapter II compared 18- and 24-month mice and found *Selh* mRNA level decreases with age in male heart, female heart, and male kidney of elderly mice. The known functions of SELH at cellular level include redox regulation, senescence suppression, UV-irradiation protection, and mitochondria biogenesis (37, 43, 46, 47). Lately, a study on genetic *Selh* deficiency in zebrafish has been reported. It shows defected organ development, activated p53 pathway, and induced inflammation in *Selh* mutant zebrafish (44).

Due to the lack of available quality antibody against SELH, its endogenous cellular expression could not be determined. However, using constructed plasmid, wild-type SELH was successfully overexpressed and Se in serum was found not enough for its full expression. One interesting finding in the second study is how overexpressed SELH impacts the expression of other selenoproteins. Decreased protein expression of GPX1 and TrxR1 is most likely due to the limited Se in the culture medium since adding Na_2SeO_3 could rescue their expressions. The protective effects against oxidative stress induced compromised proliferation and senescence are most significant when SELH

overexpression and additional Na_2SeO_3 were combined. This combined effect of SELH and additional Na_2SeO_3 suggest that when using overexpression plasmid to study selenoproteins, making sure sufficient Se is provided in the culture medium. It can support the full expression of selenoprotein when selenoprotein mRNA is produced, as well as prevent the possible decreased expression of other selenoproteins. Our study using SELH expression plasmids and various concentrations of Se showed the rational to provide additional Se when overexpression of selective selenoprotein is desired. What is more, in Chapter III, all transcriptional and translational levels were measured 36 hours after transfection. It would be interesting to extend the experiment period and see whether mRNA and protein expressions would change.

The protective effects of SELH against stress-induced senescence was studied, in the presence of additional Se. However, due to the unavailability of primary cells overexpressed SELH, the effect of SELH on replicative senescence could not be determined.

Unlike protection against oxidative stress, promotion of HSPs mRNA expression by SELH does not compromise in the absence of supplemental Se. Future studies could investigate how deficient Se or *Selh* expression would affect the expression of these chosen HSPs and whether *Selh* exerts its senescence suppression functions in an HSP-dependent or HSP-independent manner.

Two isoforms of SELH have been identified through trypsin-digested peptide sequencing. Unfortunately, the composition differences between these two proteins could not be identified. Further studies are needed to determine the specific structures and functions of the short form of SELH.

REFERENCES

1. Brown K, Arthur J. Selenium, selenoproteins and human health: a review. Public health nutrition 2001;4(2b):593-9.

2. Roman M, Jitaru P, Barbante C. Selenium biochemistry and its role for human health. Metallomics 2014;6(1):25-54.

3. Gupta UC, Gupta SC. Selenium in soils and crops, its deficiencies in livestock and humans: implications for management. Communications in Soil Science & Plant Analysis 2000;31(11-14):1791-807.

4. Fairweather-Tait SJ, Collings R, Hurst R. Selenium bioavailability: current knowledge and future research requirements. The American journal of clinical nutrition 2010;91(5):1484S-91S.

5. Dumont E, Vanhaecke F, Cornelis R. Selenium speciation from food source to metabolites: a critical review. Analytical and bioanalytical chemistry 2006;385(7):1304-23.

6. Chen X, Yang G, Chen J, Chen X, Wen Z, Ge K. Studies on the relations of selenium and Keshan disease. Biological Trace Element Research 1980;2(2):91-107.

7. Yang G, Wang S, Zhou R, Sun S. Endemic selenium intoxication of humans in China. The American journal of clinical nutrition 1983;37(5):872-81.

8. CaiXia F, JiaJun L, Shen L, RuiZhong H, GuoXiang C. Petrogenesis and sedimentary environment of the cherts from Yutangba, western Hubei Province: Evidence from silicon, oxygen, carbon and sulfur isotopic compositions. Acta Petrologica Sinica 2009;25(5):1253-9.

9. Longnecker MP, Stram DO, Taylor PR, Levander OA, Howe M, Veillon C, McAdam PA, Patterson KY, Holden JM, et al. Use of selenium concentration in whole blood, serum, toenails, or urine as a surrogate measure of selenium intake. Epidemiology 1996:384-90.

10. Rayman MP. Selenium and human health. The Lancet 2012;379(9822):1256-68.

11. Vinceti M, Dennert G, Crespi CM, Zwahlen M, Brinkman M, Zeegers M, Horneber M, D'Amico R, Del Giovane C. Selenium for preventing cancer. The Cochrane Library 2014.

12. Duffield-Lillico AJ, Reid ME, Turnbull BW, Combs GF, Jr., Slate EH, Fischbach LA, Marshall JR, Clark LC. Baseline characteristics and the effect of selenium supplementation on cancer incidence in a randomized clinical trial: a summary report of the Nutritional Prevention of Cancer Trial. Cancer Epidemiol Biomarkers Prev 2002;11(7):630-9.

13. Klein EA, Thompson IM, Jr., Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, Minasian LM, Ford LG, Parnes HL, et al. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). JAMA 2011;306(14):1549-56. doi: 10.1001/jama.2011.1437.

14. Li GX, Lee HJ, Wang Z, Hu H, Liao JD, Watts JC, Combs GF, Jr., Lu J. Superior in vivo inhibitory efficacy of methylseleninic acid against human prostate cancer over selenomethionine or selenite. Carcinogenesis 2008;29(5):1005-12.

15. Rocourt CR, Cheng W-H. Selenium supranutrition: are the potential benefits of chemoprevention outweighed by the promotion of diabetes and insulin resistance? Nutrients 2013;5(4):1349-65.

16. Riese C, Michaelis M, Mentrup B, Götz F, Köhrle J, Schweizer U, Schomburg L. Selenium-dependent pre-and posttranscriptional mechanisms are responsible for sexual dimorphic expression of selenoproteins in murine tissues. Endocrinology 2006;147(12):5883-92.

17. Turanov A, Malinouski M, Gladyshev V, Hatfield D, Berry M. Selenium: Its Molecular Biology and Role in Human Health. 2012.

18. Behne D, Weiler H, Kyriakopoulos A. Effects of selenium deficiency on testicular morphology and function in rats. Journal of reproduction and fertility 1996;106(2):291-7.

19. Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigó R, Gladyshev VN. Characterization of mammalian selenoproteomes. Science 2003;300(5624):1439-43.

20. Lee BJ, Worland PJ, Davis JN, Stadtman TC, Hatfield DL. Identification of a selenocysteyl-tRNA (Ser) in mammalian cells that recognizes the nonsense codon, UGA. Journal of Biological Chemistry 1989;264(17):9724-7.

21. Driscoll DM, Copeland PR. Mechanism and regulation of selenoprotein synthesis. Annual review of nutrition 2003;23(1):17-40.

22. Labunskyy VM, Hatfield DL, Gladyshev VN. Selenoproteins: molecular pathways and physiological roles. Physiological reviews 2014;94(3):739-77.

23. Miniard AC, Middleton LM, Budiman ME, Gerber CA, Driscoll DM. Nucleolin binds to a subset of selenoprotein mRNAs and regulates their expression. Nucleic acids research 2010;38(14):4807-20.

24. Budiman ME, Bubenik JL, Miniard AC, Middleton LM, Gerber CA, Cash A, Driscoll DM. Eukaryotic initiation factor 4a3 is a selenium-regulated RNA-binding protein that selectively inhibits selenocysteine incorporation. Molecular cell 2009;35(4):479-89.

25. Turanov AA, Everley RA, Hybsier S, Renko K, Schomburg L, Gygi SP, Hatfield DL, Gladyshev VN. Regulation of selenocysteine content of human selenoprotein P by dietary selenium and insertion of cysteine in place of selenocysteine. PloS one 2015;10(10):e0140353.

26. Xu X-M, Turanov AA, Carlson BA, Yoo M-H, Everley RA, Nandakumar R, Sorokina I, Gygi SP, Gladyshev VN, et al. Targeted insertion of cysteine by decoding UGA codons with mammalian selenocysteine machinery. Proceedings of the National Academy of Sciences 2010;107(50):21430-4.

27. Hill KE, Lyons PR, Burk RF. Differential regulation of rat liver selenoprotein mRNAs in selenium deficiency. Biochemical and biophysical research communications 1992;185(1):260-3.

28. Sunde R, Raines A, Barnes K, Evenson J. Selenium status highly regulates selenoprotein mRNA levels for only a subset of the selenoproteins in the selenoproteome. Bioscience reports 2009;29:329-38.

29. Kipp A, Banning A, van Schothorst EM, Meplan C, Schomburg L, Evelo C, Coort S, Gaj S, Keijer J, et al. Four selenoproteins, protein biosynthesis, and Wnt signalling are particularly sensitive to limited selenium intake in mouse colon. Molecular nutrition & food research 2009;53(12):1561-72.

30. Seyedali A, Berry MJ. Nonsense-mediated decay factors are involved in the regulation of selenoprotein mRNA levels during selenium deficiency. RNA 2014;20(8):1248-56.

31. Bellinger FP, Raman AV, Reeves MA, Berry MJ. Regulation and function of selenoproteins in human disease. Biochemical Journal 2009;422(1):11-22.

32. Moghadaszadeh B, Petit N, Jaillard C, Brockington M, Roy SQ, Merlini L, Romero N, Estournet B, Desguerre I, et al. Mutations in SEPN1 cause congenital muscular dystrophy with spinal rigidity and restrictive respiratory syndrome. Nature genetics 2001;29(1):17-8.

33. Dumitrescu AM, Liao XH, Abdullah MS, Lado-Abeal J, Majed FA, Moeller LC, Boran G, Schomburg L, Weiss RE, et al. Mutations in SECISBP2 result in abnormal thyroid hormone metabolism. Nature genetics 2005;37(11):1247-52.

34. Schoenmakers E, Agostini M, Mitchell C, Schoenmakers N, Papp L, Rajanayagam O, Padidela R, Ceron-Gutierrez L, Doffinger R, et al. Mutations in the selenocysteine insertion sequence–binding protein 2 gene lead to a multisystem selenoprotein deficiency disorder in humans. The Journal of clinical investigation 2010;120(12):4220-35.

35. Morozova N, Forry EP, Shahid E, Zavacki AM, Harney JW, Kraytsberg Y, Berry MJ. Antioxidant function of a novel selenoprotein in Drosophila melanogaster. Genes to Cells 2003;8(12):963-71.

36. Lobanov AV, Hatfield DL, Gladyshev VN. Eukaryotic selenoproteins and selenoproteomes. Biochimica et Biophysica Acta (BBA)-General Subjects 2009;1790(11):1424-8.

37. Novoselov SV, Kryukov GV, Xu X-M, Carlson BA, Hatfield DL, Gladyshev VN. Selenoprotein H is a nucleolar thioredoxin-like protein with a unique expression pattern. Journal of Biological Chemistry 2007;282(16):11960-8.

38. Zupanic A, Meplan C, Huguenin GV, Hesketh JE, Shanley DP. Modeling and gene knockdown to assess the contribution of nonsense-mediated decay, premature termination, and selenocysteine insertion to the selenoprotein hierarchy. RNA 2016;22(7):1076-84.

39. Legrain Y, Touat-Hamici Z, Chavatte L. Interplay between selenium levels, selenoprotein expression, and replicative senescence in WI-38 human fibroblasts. Journal of Biological Chemistry 2014;289(9):6299-310.

40. Wu RT, Cao L, Chen BP, Cheng W-H. Selenoprotein H suppresses cellular senescence through genome maintenance and redox regulation. Journal of Biological Chemistry 2014;289(49):34378-88.

41. Stoytcheva ZR, Vladimirov V, Douet V, Stoychev I, Berry MJ. Metal transcription factor-1 regulation via MREs in the transcribed regions of selenoprotein H and other metal-responsive genes. Biochimica et Biophysica Acta (BBA)-General Subjects 2010;1800(3):416-24.

42. Dikiy A, Novoselov SV, Fomenko DE, Sengupta A, Carlson BA, Cerny RL, Ginalski K, Grishin NV, Hatfield DL, et al. SelT, SelW, SelH, and Rdx12: genomics and molecular insights into the functions of selenoproteins of a novel thioredoxin-like family. Biochemistry 2007;46(23):6871-82.

43. Panee J, Stoytcheva ZR, Liu W, Berry MJ. Selenoprotein H is a redox-sensing high mobility group family DNA-binding protein that up-regulates genes involved in glutathione synthesis and phase II detoxification. Journal of Biological Chemistry 2007;282(33):23759-65.

44. Cox AG, Tsomides A, Kim AJ, Saunders D, Hwang KL, Evason KJ, Heidel J, Brown KK, Yuan M, et al. Selenoprotein H is an essential regulator of redox homeostasis that cooperates with p53 in development and tumorigenesis. Proceedings of the National Academy of Sciences 2016:201600204.

45. Jilani KEB, Panee J, He Q, Berry MJ, Li P-A. Overexpression of selenoprotein H reduces Ht22 neuronal cell death after UVB irradiation by preventing superoxide formation. International Journal of Biological Sciences 2007;3(4):198-204.

46. Mendelev N, Witherspoon S, Li PA. Overexpression of human selenoprotein H in neuronal cells ameliorates ultraviolet irradiation-induced damage by modulating cell signaling pathways. Experimental neurology 2009;220(2):328-34.

47. Mendelev N, Mehta SL, Witherspoon S, He Q, Sexton JZ, Li PA. Upregulation of human selenoprotein H in murine hippocampal neuronal cells promotes mitochondrial biogenesis and functional performance. Mitochondrion 2011;11(1):76-82.

48. Kirkwood TB, Austad SN. Why do we age? Nature 2000;408(6809):233-8.

49. Weinert BT, Timiras PS. Invited review: Theories of aging. Journal of applied physiology 2003;95(4):1706-16.

50. Hartl FU. Cellular Homeostasis and Aging. Annual Review Biochemistry 2016;85:1-4.

51. Riscuta G. Nutrigenomics at the Interface of Aging, Lifespan, and Cancer Prevention. The Journal of Nutrition 2016.

52. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 2007;8(9):729-40.

53. Sharpless NE, Sherr CJ. Forging a signature of in vivo senescence. Nature Reviews Cancer 2015;15(7):397-408.

54. Oeseburg H, de Boer RA, van Gilst WH, van der Harst P. Telomere biology in healthy aging and disease. Pflügers Archiv - European Journal of Physiology 2010;459(2):259-68.

55. Donate LE, Blasco MA. Telomeres in cancer and ageing. Philosophical Transactions of the Royal Society of London B: Biological Sciences 2011;366(1561):76-84.

56. Chen C-H, Chen R-J. Prevalence of telomerase activity in human cancer. Journal of the formosan medical association 2011;110(5):275-89.

57. Cesare AJ, Reddel RR. Alternative lengthening of telomeres: models, mechanisms and implications. Nature reviews genetics 2010;11(5):319-30.

58. Blasco MA, Lee H-W, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. Cell 1997;91(1):25-34.

59. Shay JW, Wright W. Telomere dynamics in cancer progression and prevention: fundamental differences in human and mouse telomere biology. Nature medicine 2000;6:849-51.

60. Lévy BI. Artery changes with aging: degeneration or adaptation. Dialogues in Cardiovascular Medicine 2001;6:104-11.

61. Buckwalter JA. Aging and degeneration of the human intervertebral disc. Spine 1995;20(11):1307-14.

62. Morrison JH, Hof PR. Life and death of neurons in the aging brain. Science 1997;278(5337):412-9.

63. Zhou R-P, Wu X-S, Wang Z-S, Xie Y-Y, Ge J-F, Chen F-H. Novel Insights into Acid-Sensing Ion Channels: Implications for Degenerative Diseases. Aging and Disease 2016;7(4):491-501.

64. Kronenberg F. Genome-wide association studies in aging-related processes such as diabetes mellitus, atherosclerosis and cancer. Experimental gerontology 2008;43(1):39-43.

65. Wu RT, Cao L, Mattson E, Witwer KW, Cao J, Zeng H, He X, Combs GFJ, Cheng W-H. Opposing impacts on healthspan and longevity by limiting dietary selenium in telomere dysfunctional mice. Aging cell 2016;10.1111/acel.12529.

66. McCann JC, Ames BN. Adaptive dysfunction of selenoproteins from the perspective of the triage theory: why modest selenium deficiency may increase risk of diseases of aging. The FASEB Journal 2011;25(6):1793-814.

67. Ogawa-Wong AN, Berry MJ, Seale LA. Selenium and metabolic disorders: An emphasis on type 2 diabetes risk. Nutrients 2016;8(2):80.

68. Hwang DY, Cho JS, Oh JH, Shim SB, Jee SW, Lee SH, Seo SJ, Lee S-K, Lee SH, et al. Differentially expressed genes in transgenic mice carrying human mutant presenilin-2 (N141I): Correlation of selenoprotein M with Alzheimer's disease. Neurochemical research 2005;30(8):1009-19.

69. Takemoto AS, Berry MJ, Bellinger FP. Role of selenoprotein P in Alzheimer's disease. Ethnicity & disease 2010;20(1 Suppl 1):S1.

70. Berry MJ, Banu L, Chen Y, Mandel SJ, Kieffer JD, Harney JW, Larsen PR. Recognition of UGA as a selenocysteine codon in type I deiodinase requires sequences in the 3′ untranslated region. Nature 1991;353(6341):273-6.

71. Behne D, Hofer T, von Berswordt-Wallrabe R, Elger W. Selenium in the testis of the rat: studies on its regulation and its importance for the organism. The Journal of nutrition 1982;112(9):1682-7.

72. Yeh J-Y, Vendeland SC, Gu Q-p, Butler JA, Ou B-R, Whanger PD. Dietary selenium increases selenoprotein W levels in rat tissues. The Journal of nutrition 1997;127(11):2165-72.

73. Pitts MW, Kremer PM, Hashimoto AC, Torres DJ, Byrns CN, Williams CS, Berry MJ. Competition between the Brain and Testes under Selenium-Compromised Conditions: Insight into Sex Differences in Selenium Metabolism and Risk of Neurodevelopmental Disease. The Journal of Neuroscience 2015;35(46):15326-38.

74. Schomburg L, Schweizer U. Hierarchical regulation of selenoprotein expression and sex-specific effects of selenium. Biochimica et Biophysica Acta (BBA) - General Subjects 2009;1790(11):1453-62.

75. Ursini F, Heim S, Kiess M, Maiorino M, Roveri A, Wissing J, Flohé L. Dual function of the selenoprotein PHGPx during sperm maturation. Science 1999;285(5432):1393-6.

76. Schomburg L, Riese C, Renko K, Schweizer U. Effect of age on sexually dimorphic selenoprotein expression in mice. Biological chemistry 2007;388(10):1035-41.

77. Akbaraly NT, Hininger-Favier I, Carriere I, Arnaud J, Gourlet V, Roussel A-M, Berr C. Plasma selenium over time and cognitive decline in the elderly. Epidemiology 2007;18(1):52-8.

78. Collins K. Mammalian telomeres and telomerase. Current opinion in cell biology 2000;12(3):378-83.

79. Von Zglinicki T, Saretzki G, Ladhoff J, di Fagagna FdA, Jackson S. Human cell senescence as a DNA damage response. Mechanisms of ageing and development 2005;126(1):111-7.

80. González-Suárez E, Goytisolo FA, Flores JM, Blasco MA. Telomere dysfunction results in enhanced organismal sensitivity to the alkylating agent N-methyl-N-nitrosourea. Cancer research 2003;63(21):7047-50.

81. Herrera E, Samper E, Martín‐Caballero J, Flores JM, Lee HW, Blasco MA. Disease states associated with telomerase deficiency appear earlier in mice with short telomeres. The EMBO Journal 1999;18(11):2950-60.

82. Olivieri O, Stanzial AM, Girelli D, Trevisan MT, Guarini P, Terzi M, Caffi S, Fontana F, Casaril M, et al. Selenium status, fatty acids, vitamins A and E, and aging: the Nove Study. The American journal of clinical nutrition 1994;60(4):510-7.

83. Ho Y-S, Magnenat J-L, Bronson RT, Cao J, Gargano M, Sugawara M, Funk CD. Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. Journal of Biological Chemistry 1997;272(26):16644-51.

84. de Haan JB, Bladier C, Lotfi-Miri M, Taylor J, Hutchinson P, Crack PJ, Hertzog P, Kola I. Fibroblasts derived from Gpx1 knockout mice display senescent-like features and are susceptible to H 2 O 2-mediated cell death. Free Radical Biology and Medicine 2004;36(1):53-64.

85. Holmstrom A, Ryan T, Zeng H, Lei K, Cheng W-H. Nutritional and supranutritional levels of selenate differentially suppress prostate tumor growth in adult but not young nude mice. The Journal of nutritional biochemistry 2012;23(9):1086-91.

86. Flurkey K, Currer J, Harrison D. Mouse models in aging research. 2007.

87. Davis CD, Zeng H, Finley JW. Selenium-enriched broccoli decreases intestinal tumorigenesis in multiple intestinal neoplasia mice. The Journal of nutrition 2002;132(2):307-9.

88. Sun L-H, Li J-G, Zhao H, Shi J, Huang J-Q, Wang K-N, Xia X-J, Li L, Lei XG. Porcine serum can be biofortified with selenium to inhibit proliferation of three types of human cancer cells. The Journal of nutrition 2013;143(7):1115-22.

89. Liu Y, Zhao H, Zhang Q, Tang J, Li K, Xia X-J, Wang K-N, Li K, Lei XG. Prolonged dietary selenium deficiency or excess does not globally affect selenoprotein gene expression and/or protein production in various tissues of pigs. The Journal of nutrition 2012;142(8):1410-6.

90. Zhou J-C, Zhao H, Li J-G, Xia X-J, Wang K-N, Zhang Y-J, Liu Y, Zhao Y, Lei XG. Selenoprotein gene expression in thyroid and pituitary of young pigs is not affected by dietary selenium deficiency or excess. The Journal of nutrition 2009;139(6):1061-6.

91. Huang J-Q, Li D-L, Zhao H, Sun L-H, Xia X-J, Wang K-N, Luo X, Lei XG. The selenium deficiency disease exudative diathesis in chicks is associated with downregulation of seven common selenoprotein genes in liver and muscle. The Journal of nutrition 2011;141(9):1605-10.

92. Sunde RA, Raines AM. Selenium regulation of the selenoprotein and nonselenoprotein transcriptomes in rodents. Advances in Nutrition 2011;2(2):138-50.

93. Austad SN, Fischer KE. Sex Differences in Lifespan. Cell Metabolism 2016;23(6):1022-33.

94. Min K-J, Lee C-K, Park H-N. The lifespan of Korean eunuchs. Current Biology 2012;22(18):R792-R3.

95. Hamilton JB, Mestler GE. Mortality and survival: comparison of eunuchs with intact men and women in a mentally retarded population. Journal of Gerontology 1969;24(4):395-411.

96. Harrison DE, Strong R, Allison DB, Ames BN, Astle CM, Atamna H, Fernandez E, Flurkey K, Javors MA, et al. Acarbose, 17‐α‐estradiol, and nordihydroguaiaretic acid extend mouse lifespan preferentially in males. Aging cell 2014;13(2):273-82.

97. Kaushik S, Cuervo AM. Proteostasis and aging. Nature medicine 2015;21(12):1406-15.

98. Arbogast S, Ferreiro A. Selenoproteins and protection against oxidative stress: selenoprotein N as a novel player at the crossroads of redox signaling and calcium homeostasis. Antioxidants & redox signaling 2010;12(7):893-904.

99. Castets P, Bertrand AT, Beuvin M, Ferry A, Le Grand F, Castets M, Chazot G, Rederstorff M, Krol A, et al. Satellite cell loss and impaired muscle regeneration in selenoprotein N deficiency. Human molecular genetics 2011;20(4):694-704.

100. Ferreiro A, Quijano-Roy S, Pichereau C, Moghadaszadeh B, Goemans N, Bönnemann C, Jungbluth H, Straub V, Villanova M, et al. Mutations of the selenoprotein N gene, which is implicated in rigid spine muscular dystrophy, cause the classical phenotype of multiminicore disease: reassessing the nosology of early-onset myopathies. The American Journal of Human Genetics 2002;71(4):739-49.

101. Rederstorff M, Castets P, Arbogast S, Lainé J, Vassilopoulos S, Beuvin M, Dubourg O, Vignaud A, Ferry A, et al. Increased muscle stress-sensitivity induced by selenoprotein N inactivation in mouse: a mammalian model for SEPN1-related myopathy. PloS One 2011;6(8):e23094.

102. Zeöld A, Doleschall M, Haffner MC, Capelo LP, Menyhért J, Liposits Z, da Silva WS, Bianco AC, Kacskovics I, et al. Characterization of the Nuclear Factor-κB Responsiveness of the Human dio 2 Gene. Endocrinology 2006;147(9):4419-29.

103. Prabhu KS, Zamamiri-Davis F, Stewart JB, Thompson JT, Sordillo LM, Reddy CC. Selenium deficiency increases the expression of inducible nitric oxide synthase in RAW 264.7 macrophages: role of nuclear factor-κB in up-regulation. Biochemical Journal 2002;366(1):203-9.

104. Zhang J-l, Zhang Z-W, Shan A-S, Xu S-w. Effects of dietary selenium deficiency or excess on gene expression of selenoprotein N in chicken muscle tissues. Biological trace element research 2014;157(3):234-41.

105. Maciel‐Dominguez A, Swan D, Ford D, Hesketh J. Selenium alters miRNA profile in an intestinal cell line: Evidence that miR‐185 regulates expression of GPX2 and SEPSH2. Molecular nutrition & food research 2013;57(12):2195-205.

106. St. Germain DL, Galton VA, Hernandez A. Defining the roles of the iodothyronine deiodinases: current concepts and challenges. Endocrinology 2009;150(3):1097-107.

107. Castillo M, Hall JA, Correa-Medina M, Ueta C, Kang HW, Cohen DE, Bianco AC. Disruption of thyroid hormone activation in type 2 deiodinase knockout mice causes obesity with glucose intolerance and liver steatosis only at thermoneutrality. Diabetes 2011;60(4):1082-9.

108. Marsili A, Aguayo-Mazzucato C, Chen T, Kumar A, Chung M, Lunsford EP, Harney JW, Van-Tran T, Gianetti E, et al. Mice with a targeted deletion of the type 2 deiodinase are insulin resistant and susceptible to diet induced obesity. PLoS One 2011;6(6):e20832.

109. Labunskyy VM, Lee BC, Handy DE, Loscalzo J, Hatfield DL, Gladyshev VN. Both maximal expression of selenoproteins and selenoprotein deficiency can promote development of type 2 diabetes-like phenotype in mice. Antioxidants & redox signaling 2011;14(12):2327-36.

110. Moustafa ME, Carlson BA, El-Saadani MA, Kryukov GV, Sun Q-A, Harney JW, Hill KE, Combs GF, Feigenbaum L, et al. Selective inhibition of selenocysteine tRNA maturation and selenoprotein synthesis in transgenic mice expressing isopentenyladenosine-deficient selenocysteine tRNA. Molecular and cellular biology 2001;21(11):3840-52.

111. Singh A, Rangasamy T, Thimmulappa RK, Lee H, Osburn WO, Brigelius-Flohé R, Kensler TW, Yamamoto M, Biswal S. Glutathione peroxidase 2, the major cigarette smoke–inducible isoform of GPX in lungs, is regulated by Nrf2. American journal of respiratory cell and molecular biology 2006;35(6):639-50.

112. Gao Y, Hannan NR, Wanyonyi S, Konstantopolous N, Pagnon J, Feng HC, Jowett JB, Kim K-H, Walder K, et al. Activation of the selenoprotein SEPS1 gene expression by pro-inflammatory cytokines in HepG2 cells. Cytokine 2006;33(5):246-51.

113. Zucker SN, Fink EE, Bagati A, Mannava S, Bianchi-Smiraglia A, Bogner PN, Wawrzyniak JA, Foley C, Leonova KI, et al. Nrf2 amplifies oxidative stress via induction of Klf9. Molecular cell 2014;53(6):916-28.

114. Su D, Novoselov SV, Sun Q-A, Moustafa ME, Zhou Y, Oko R, Hatfield DL, Gladyshev VN. Mammalian selenoprotein thioredoxin-glutathione reductase roles in disulfide bond formation and sperm maturation. Journal of Biological Chemistry 2005;280(28):26491-8.

115. Mariotti M, Ridge PG, Zhang Y, Lobanov AV, Pringle TH, Guigo R, Hatfield DL, Gladyshev VN. Composition and evolution of the vertebrate and mammalian selenoproteomes. PLoS One 2012;7(3):e33066.

116. Wu A, Oldfield J, Shull L, Cheeke P. Specific effect of selenium deficiency on rat sperm. Biology of reproduction 1979;20(4):793-8.

117. Hill KE, Zhou J, McMahan WJ, Motley AK, Atkins JF, Gesteland RF, Burk RF. Deletion of selenoprotein P alters distribution of selenium in the mouse. Journal of Biological Chemistry 2003;278(16):13640-6.

118. Olson GE, Winfrey VP, NagDas SK, Hill KE, Burk RF. Apolipoprotein E receptor-2 (ApoER2) mediates selenium uptake from selenoprotein P by the mouse testis. Journal of Biological Chemistry 2007;282(16):12290-7.

119. Imai H, Hakkaku N, Iwamoto R, Suzuki J, Suzuki T, Tajima Y, Konishi K, Minami S, Ichinose S, et al. Depletion of selenoprotein GPx4 in spermatocytes causes male infertility in mice. Journal of Biological Chemistry 2009;284(47):32522-32.

120. Schneider M, Förster H, Boersma A, Seiler A, Wehnes H, Sinowatz F, Neumüller C, Deutsch MJ, Walch A, et al. Mitochondrial glutathione peroxidase 4 disruption causes male infertility. The FASEB journal 2009;23(9):3233-42.

121. Arnaud J, Bertrais S, Roussel A, Arnault N, Ruffieux D, Favier A, Berthelin S, Estaquio C, Galan P, et al. Serum selenium determinants in French adults: the SU. VI. M. AX study. British Journal of Nutrition 2006;95(02):313-20.

122. Wu Q, Huang K. Effect of long-term Se deficiency on the antioxidant capacities of rat vascular tissue. Biological trace element research 2004;98(1):73-84.

123. Barnes KM, Evenson JK, Raines AM, Sunde RA. Transcript analysis of the selenoproteome indicates that dietary selenium requirements of rats based on selenium-regulated selenoprotein mRNA levels are uniformly less than those based on glutathione peroxidase activity. The Journal of nutrition 2009;139(2):199-206.

124. Hoefig CS, Renko K, Köhrle J, Birringer M, Schomburg L. Comparison of different selenocompounds with respect to nutritional value vs. toxicity using liver cells in culture. The Journal of nutritional biochemistry 2011;22(10):945-55.

125. Wu M, Kang MM, Schoene NW, Cheng W-H. Selenium compounds activate early barriers of tumorigenesis. Journal of Biological Chemistry 2010;285(16):12055-62.

126. Takac T, Vadovic P, Pechan T, Luptovciak I, Samajova O, Samaj J. Comparative proteomic study of Arabidopsis mutants mpk4 and mpk6. Scientific Reports 2016;6:28306.

127. Karlenius TC, Shah F, Yu W-C, Hawkes H-J, Tinggi U, Clarke FM, Tonissen KF. The selenium content of cell culture serum influences redox-regulated gene expression. Biotechniques 2011;50(5):295-301.

128. Yi J, Luo J. SIRT1 and p53, effect on cancer, senescence and beyond. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 2010;1804(8):1684-9.

129. Lee J-S, Surh Y-J. Nrf2 as a novel molecular target for chemoprevention. Cancer letters 2005;224(2):171-84.

130. Oesterreich S, Hickey E, Weber LA, Fuqua SA. Basal regulatory promoter elements of the hsp27 gene in human breast cancer cells. Biochemical and biophysical research communications 1996;222(1):155-63.

131. Abravaya K, Phillips B, Morimoto RI. Heat shock-induced interactions of heat shock transcription factor and the human hsp70 promoter examined by in vivo footprinting. Molecular and cellular biology 1991;11(1):586-92.

132. Mathur SK, Sistonen L, Brown IR, Murphy SP, Sarge KD, Morimoto RI. Deficient induction of human hsp70 heat shock gene transcription in Y79 retinoblastoma cells despite activation of heat shock factor 1. Proceedings of the National Academy of Sciences 1994;91(18):8695-9.

133. Wilkerson DC, Skaggs HS, Sarge KD. HSF2 binds to the Hsp90, Hsp27, and c-Fos promoters constitutively and modulates their expression. Cell stress & chaperones 2007;12(3):283-90.

134. Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. Nature 2003;426(6968):895-9.

135. Lydeard JR, Schulman BA, Harper JW. Building and remodelling Cullin–RING E3 ubiquitin ligases. EMBO reports 2013;14(12):1050-61.

136. Lin H-C, Ho S-C, Chen Y-Y, Khoo K-H, Hsu P-H, Yen H-CS. CRL2 aids elimination of truncated selenoproteins produced by failed UGA/Sec decoding. Science 2015;349(6243):91-5.

137. Yamada K, Kawata H, Zhangfei S, Hirano S, Mizutani T, Yazawa T, Sekiguchi T, Yoshino M, Kajitani T, et al. Analysis of zinc-fingers and homeoboxes (ZHX)-1-interacting proteins: molecular cloning and characterization of a member of the ZHX family, ZHX3. Biochemical Journal 2003;373(1):167-78.

138. Wilkie GS, Dickson KS, Gray NK. Regulation of mRNA translation by 5′-and 3′-UTR-binding factors. Trends in biochemical sciences 2003;28(4):182-8.

139. Lee I, Ajay SS, Yook JI, Kim HS, Hong SH, Kim NH, Dhanasekaran SM, Chinnaiyan AM, Athey BD. New class of microRNA targets containing simultaneous 5′-UTR and 3′-UTR interaction sites. Genome research 2009;19(7):1175-83.

140. Popp MW-L, Maquat LE. Organizing principles of mammalian nonsense-mediated mRNA decay. Annual review of genetics 2013;47:139.

141. Schweingruber C, Rufener SC, Zünd D, Yamashita A, Mühlemann O. Nonsense-mediated mRNA decay—mechanisms of substrate mRNA recognition and degradation in mammalian cells. Biochimica Et Biophysica Acta (BBA)-Gene Regulatory Mechanisms 2013;1829(6):612-23.

142. Handy DE, Zhang Y, Loscalzo J. Homocysteine down-regulates cellular glutathione peroxidase (GPx1) by decreasing translation. Journal of Biological Chemistry 2005;280(16):15518-25.

143. Florian S, Krehl S, Loewinger M, Kipp A, Banning A, Esworthy S, Chu F-F, Brigelius-Flohé R. Loss of GPx2 increases apoptosis, mitosis, and GPx1 expression in the intestine of mice. Free Radical Biology and Medicine 2010;49(11):1694-702.

144. Zhang J, Švehlíková V, Bao Y, Howie AF, Beckett GJ, Williamson G. Synergy between sulforaphane and selenium in the induction of thioredoxin reductase 1 requires both transcriptional and translational modulation. Carcinogenesis 2003;24(3):497-503.

145. Veal EA, Day AM, Morgan BA. Hydrogen peroxide sensing and signaling. Molecular cell 2007;26(1):1-14.

146. de Haan JB, Bladier C, Griffiths P, Kelner M, O'Shea RD, Cheung NS, Bronson R, Silvestro MJ, Wild S, et al. Mice with a homozygous null mutation for the most abundant glutathione peroxidase, Gpx1, show increased susceptibility to the oxidative stress-inducing agents paraquat and hydrogen peroxide. Journal of Biological Chemistry 1998;273(35):22528-36.

147. Kabuyama Y, Kitamura T, Yamaki J, Homma MK, Kikuchi S-i, Homma Y. Involvement of thioredoxin reductase 1 in the regulation of redox balance and viability of rheumatoid synovial cells. Biochemical and biophysical research communications 2008;367(2):491-6.

148. Volonte D, Galbiati F. Inhibition of thioredoxin reductase 1 by caveolin 1 promotes stress‐induced premature senescence. EMBO reports 2009;10(12):1334-40.

149. Kregel KC. Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. Journal of applied physiology 2002;92(5):2177-86.

150. Chen X, Zhu Y-H, Cheng X-Y, Zhang Z-W, Xu S-W. The protection of selenium against cadmium-induced cytotoxicity via the heat shock protein pathway in chicken splenic lymphocytes. Molecules 2012;17(12):14565-72.

151. Manikandan R, Beulaja M, Thiagarajan R, Arumugam M. Effect of curcumin on the modulation of alphaA- and alphaB-crystallin and heat shock protein 70 in selenium-induced cataractogenesis in Wistar rat pups. Molecular Vision 2011;17:388-94.

152. Calderwood SK, Murshid A, Prince T. The shock of aging: molecular chaperones and the heat shock response in longevity and aging–a mini-review. Gerontology 2009;55(5):550-8.

153. Kurapati R, Passananti HB, Rose MR, Tower J. Increased hsp22 RNA levels in Drosophila lines genetically selected for increased longevity. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 2000;55(11):B552-B9.

154. Yokoyama K, Fukumoto K, Murakami T, Harada S-i, Hosono R, Wadhwa R, Mitsui Y, Ohkuma S. Extended longevity of Caenorhabditis elegans by knocking in extra copies of hsp70F, a homolog of mot‐2 (mortalin)/mthsp70/Grp75. FEBS letters 2002;516(1-3):53-7.

155. Wyttenbach A, Sauvageot O, Carmichael J, Diaz-Latoud C, Arrigo A-P, Rubinsztein DC. Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. Human molecular genetics 2002;11(9):1137-51.

156. Liang X, Fomenko DE, Hua D, Kaya A, Gladyshev VN. Diversity of protein and mRNA forms of mammalian methionine sulfoxide reductase B1 due to intronization and protein processing. PloS one 2010;5(7):e11497.

157. Ewan RC. Effect of selenium on rat growth, growth hormone and diet utilization. The Journal of nutrition 1976;106(5):702-9.

158. Kasaikina MV, Kravtsova MA, Lee BC, Seravalli J, Peterson DA, Walter J, Legge R, Benson AK, Hatfield DL, et al. Dietary selenium affects host selenoproteome expression by influencing the gut microbiota. The FASEB Journal 2011;25(7):2492-9.

APPENDIX A

LIST OF ABBREVIATION

full name	abbreviated
ALT	alternative lengthening of telomeres
CRL	cullin-RING ubiquitin ligases
Dio	Deionidase
DMEM	Dulbecco's Modified Eagle Medium
EV	empty vector
FBS	fetal bovine serum
FLAG	Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys
Gpx	Glutathione Peroxidase
GSH	glutathione
HSE	heat shock element
HSP	heat shock protein
HSP27	heat shock protein 27
HSP70-1A	heat shock 70 kDa protein 1A
HSP70-1B	heat shock 70 kDa protein 1B
HSP90AA!	heat shock protein 90 alpha family class A member 1
MsrB1	Methionine Sulfoxide Reductase B1
NMD	nonsense-mediated decay
NRF2	Nuclear factor (erythroid-derived 2)-like 2
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
SECIS	selenocysteine insertion sequence
SBP2	SECIS-Binding Protein 2
Se	Selenium
Sec	Selenocysteine
Se-Met	Selenomethionine
Sephs2	Selenophosphate Synthetase 2
Sel15	Selenoprotein 15
Selh	Selenoprotein H
Seli	Selenoprotein I
Selk	Selenoprotein K
Selm	Selenoprotein M

Seln	Selenoprotein N
Selo	Selenoprotein O
Sepp1	Selenoprotein P
Sels	Selenoprotein S
Selt	Selenoprotein T
Selv	Selenoprotein V
Selw	Selenoprotein W
Txnrd1 /TrxR1	Thioredoxin Reductase 1
Txnrd2 /TrxR3	Thioredoxin Reductase 2
Txnrd3	Thioredoxin Reductase 3
tRNA	transfer RNA
Trx	thioredoxin
UTR	untranslated region